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| <p>(54) Title: METHOD FOR REDUCTION OF TRANSGENE COPY NUMBER</p> <p>(57) Abstract</p> <p>The present invention provides methods for the efficient production of transformants with low transgene copy numbers. In the method, the average transgene copy number of transformants is decreased through methods which are believed to limit the interaction between segments of transforming DNA prior to transformation. The methods comprise means for end-modification of transforming DNA and use of limited quantities of DNA for transformation. Production of single or low copy transformation events is desirable in that it avoids many of the problems associated with high transgene copy number including co-suppression, unpredictable gene expression and non-Mendelian inheritance.</p> | | | |

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DESCRIPTION

METHOD FOR REDUCTION OF TRANSGENE COPY NUMBER

1. Field of the Invention

5 The present invention relates generally to the field of plant transformation. More particularly, it concerns methods for reducing the transgene copy number of transformants.

2. Description of Related Art

10 Recent advances in molecular biology have dramatically expanded the ability of scientists to manipulate the germplasm of animals and plants. Genes controlling specific phenotypes, for example, specific polypeptides that lend insect, antibiotic and herbicide resistance, have been identified in certain germplasm and isolated therefrom. Even more important has been the ability to introduce these genes into a heterologous organism. This transformation may be accomplished even where the recipient organism is from a different 15 phylum, genus or species from that which donated the gene (heterologous transformation).

Many attempts have been made to genetically engineer desired traits into plant genomes by introduction of exogenous genes using genetic engineering techniques. The uptake of new DNA by recipient plant cells has been accomplished by various means, including 20 *Agrobacterium* infection (Nester *et al.*, 1984), polyethylene glycol (PEG)-mediated DNA uptake (Lorz *et al.*, 1985), electroporation of protoplasts (Fromm *et al.*, 1986) and microprojectile bombardment (Klein *et al.*, 1987).

Microprojectile bombardment is a particularly advantageous transformation technique in that, in addition to being an effective means of reproducibly stably transforming monocots, neither the isolation of protoplasts (Cristou *et al.*, 1988) nor the susceptibility to *Agrobacterium* 25 infection is required (see, for example, Gordon-Kamm *et. al.* 1990). While successful recovery of fertile transgenic plants has been accomplished in many instances by microprojectile bombardment (Fromm *et al.*, 1990; Gordon-Kamm *et al.*, 1990; Walters *et al.*, 1992), efforts have continued to optimize the efficiency of transformation. Parameters which have been suggested to be important include particle size relative to the recipient cell, particle number per 30 cell, the quantity and timing of DNA delivery, how the cells involved in transformation react to

DNA particle bombardment and limitation of physical trauma to those cells (Klein *et al.*, 1988a, 1988b.; Russell *et al.*, 1992; Vain *et al.*, 1993; Hunold *et al.*, 1994).

One difficulty associated with microprojectile bombardment transformation and other transformation systems has been the relative inability to control the copy number of introduced transgenes. Specifically, using previous transformation techniques, many transformants are produced with multiple transgene copies. High to moderate copy number transformation events are non-desirable from a number of standpoints. For example, multiple-copies of a transgene can cause co-suppression, thereby "turning off" expression of the transgene. Even in the absence of co-suppression, expression may be limited and may vary over generations. Additionally, recombination can occur between multiple gene copies leading to rearrangements in the transgenes. Complex transformation events may also exhibit non-Mendelian inheritance, greatly complicating efforts to breed transformants.

Even using traditional transformation techniques, some single copy number transformants are produced, but the relative proportion of these is typically low. When using microprojectile bombardment, frequently less than 5% of the transformants recovered posses a single transgene copy. Therefore, in order to obtain the desired single copy transformants, current technology necessitates the screening of large numbers of transformants using labor-intensive screening techniques such as Southern hybridization. This screening represents a significant burden upon scientists, and generally slows efforts in the production of novel transformants having value to the consumer. There is, therefore, a great need in the art to identify specific methods and compositions which may be used to efficiently produce low-copy transformants.

SUMMARY OF THE INVENTION

One embodiment of the current invention provides a method of preparing a transgenic plant comprising the steps of : end-modifying a plurality of nucleic acid segments, contacting recipient plant cells with said nucleic acid segments, and regenerating a plant from a plant cell which has been stably transformed with at least a first nucleic acid segment. The regenerated transgenic plants may be fertile, and the nucleic acid segments may be DNA segments.

In particular embodiments of the invention, the end-modifying of nucleic acid segments comprises dephosphorylation. The dephosphorylating may comprise treatment with any

compound capable of removing a phosphate group from nucleic acids, for example, phosphatases such as calf or bacterial alkaline phosphatase, or a phosphatase from *Aspergillus niger* or *Saccharomyces cerevisiae*. The transformed plants may be a monocot plant such as rice, wheat, barley, oats, rye, millet, sorghum, sugarcane, turfgrass and particularly, maize.

5 Where the monocot is maize, microprojectile bombardment may comprise a particularly useful method of transformation. The transformed plant may also be a dicot, for example, cotton, tomato, potato, citrus, tobacco, and particularly, soybean.

In another embodiment of the invention, transforming DNA may be end-modified by blunting the ends of the nucleic acid segments. One preferred means for said blunting comprises treating nucleic acid segments with a DNA polymerase, for example DNA Polymerase I and more preferably, the Klenow fragment of *E. coli* DNA Polymerase I. The DNA polymerase may also be T₄ DNA Polymerase. The blunting of the transforming nucleic acids also can be carried out by treating the nucleic acid segments with a nuclease capable of degrading single-stranded DNA. Preferably, a nuclease will be used which can selectively degrade single stranded DNA such as, for example, mung bean nuclease or S1 nuclease. Alternatively, the blunting may comprise treating DNA segments with a blunt-cutting restriction endonuclease.

The recipient plant cells to be transformed with blunted nucleic acids may be potentially any monocot or dicot plant. Where the cells are from a monocot plant, preferred plants will include rice, wheat, barley, oats, rye, millet, sorghum, sugarcane, turfgrass and particularly, maize. Where the recipient plant cells are from a dicot plant, preferred will be cotton, tomato, potato, citrus, tobacco and especially soybean plants. The method of transformation may comprise microprojectile bombardment, PEG-mediated transformation, or electroporation. The microprojectile bombardment may comprise coating microprojectiles with the blunted DNA segments and contacting recipient plant cells with the microprojectiles. When the recipient plant cells are maize cells, microprojectile bombardment may be preferred in particular embodiments.

In certain embodiments of the invention, the DNA segments may comprise plasmids, or preferably, an expression cassette isolated from a plasmid. The DNA segments used for transformation with any of the methods of the invention may comprise an exogenous gene encoding a selected trait. The DNA segments may still further comprise a promoter and 3' region operatively linked to the exogenous gene, and also may include a second, third, fourth,

fifth, sixth, or any additional number of exogenous genes which can physically be placed into a single DNA molecule and be used for transformation. The DNA segments comprising the exogenous gene or genes may also include a selectable or screenable marker gene. The method of transformation may, in particular embodiments, comprise electroporation, PEG-mediated transformation, microprojectile bombardment, or any other similar technique in which the methods of the invention may be used to reduce average transgene copy number.

Sill, yet another embodiment of the current invention provides a method of transforming a plant comprising the steps of preparing a microprojectile composition comprising from 1 ng to 2000 ng of transforming DNA per each 1.8 mg of starting microprojectiles; contacting recipient plant cells with said microprojectile composition; regenerating plants from recipient cells which have received said DNA; and identifying a fertile transgenic plant the genome of which has been augmented relative to that of the corresponding nontransgenic recipient plant through the stable introduction of said DNA. In particular embodiments of the invention, the microprojectile composition may be further defined as comprising 2.5 ng to 1000 ng of DNA per each 1.8 mg of starting microprojectiles; or more preferably from 2.5 ng to 750 ng of DNA per each 1.8 mg of starting microprojectiles, or more preferably from 2.5 ng to 500 ng of DNA per each 1.8 mg of starting microprojectiles, or more preferably from 2.5 ng to 250 ng of DNA per each 1.8 mg of starting microprojectiles, or even more preferably from 2.5 ng to 100 ng of DNA per each 1.8 mg of starting microprojectiles, or even more preferably from 2.5 ng to 50 ng of DNA per each 1.8 mg of starting microprojectiles.

The transforming DNA used may, in particular embodiments of the invention, comprise plasmids, or an expression cassette isolated therefrom. The DNA may comprises an exogenous gene encoding a selected trait, and still further, may include a promoter and 3' region operatively linked to the exogenous gene. The DNA segment may additionally include a second, third, fourth, fifth, sixth, or any additional number of exogenous genes capable of being placed on a single DNA molecule and transformed into a recipient cell. In particular embodiments of the invention, the DNA segments will include a selectable or screenable marker gene.

The recipient plant cells for transformation with the microprojectile bombardment compositions of the invention may be from potentially any transformable monocot or dicot plant. Preferred monocot plant cells for use with the invention are from rice, wheat, barley, oats, rye, millet, sorghum, sugarcane, turfgrass and particularly, maize. Preferred dicot plant

cells for use with the invention include cotton, tomato, potato, citrus, tobacco, and particularly soybean.

BRIEF DESCRIPTION OF THE DRAWINGS

5 The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

10 **FIG. 1.** Restriction map of plasmid pDPG570. The 35S/4XOCS-Sh1 leader/intron1-MZTP-HD73 Bt-pin3: 35S-*bar*-Tr7 cassette can be liberated from the plasmid by digestion with *No*I.

15 **FIG. 2.** Restriction map of plasmid pDPG756. The ActinP/I-lox5-*bar*-Tr7 cassette is liberated from the plasmid by digestion with *Hind*III and *Sna*B1, and with *Scal*I to digest the vector backbone into fragments smaller than the cassette.

15 DETAILED DESCRIPTION OF THE INVENTION

The present invention overcomes deficiencies in the prior art by providing methods for the efficient production of transformants with low transgene copy numbers. Specifically, the invention relates to reducing transgene copy number by dephosphorylating and/or blunting DNA prior to its use in transformation. The invention also relates to reduction of transgene copy number by the use of specific concentrations of transforming DNA. In particular embodiments of the invention, reduction of transgene copy number comprises treating transforming DNA with a DNA polymerase, single strand nuclease, or dephosphorylating enzyme. Illustrative embodiments of the foregoing include, for example, the Klenow fragment of *E. coli* DNA polymerase I, mung bean nuclease, and calf intestinal or bacterial alkaline phosphatase, respectfully. In a preferred embodiment of the invention, transformation is achieved by electroporation, PEG-mediated direct DNA uptake, and particularly, microprojectile bombardment and the like. Exemplary particle types for use with the current invention include those comprised of tungsten, gold, and platinum. In particular embodiments, gold particles are preferred for use with the current invention.

I. Control of Transgene Copy Number with the Current Invention

The current invention provides methods for decreasing the average transgene copy number in transformants by limiting the interaction of transforming DNA segments prior to integration into the host genome. Particularly, the invention limits the ability of transforming DNA to rearrange or concatamerize prior to transformation, thereby decreasing the likelihood of creating transformation events consisting of multiple and/or rearranged transgene copies.

5 One embodiment of the current invention provides methods for decreasing the interaction between molecules of transforming nucleic acid segments by end-modifying the segments. As used herein, the term "end-modifying" refers to the treatment of a nucleic acid with a composition which will blunt the ends of a nucleic acid segment or will cause the removal of a phosphate group from the nucleic acid. "Nucleic acid segments" as used herein, refers to the nucleic acids used for transformation. A preferred nucleic acid segment for use with the current invention comprises an expression cassette. Another embodiment of the current invention comprises reducing the interactions between transforming DNA molecules through the use of 10 extremely low quantities of DNA for transformation.

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The recipient cells used with the current invention may potentially be from any organism which can be transformed with the methods of the invention. Preferred recipient organisms include dicot plants such as cotton, soybeans, tomato, potato, citrus and tobacco and the monocot plants wheat, sorghum, rye, millet, sugarcane, oats, barley, turf grass and maize.

20 In particular embodiments of the invention, maize is most preferred for transformation.

(i) *Dephosphorylation of Transforming DNA*

One embodiment of the current invention provides a method of reducing the average transgene copy number of transformants by dephosphorylating DNA prior to transformation. As used herein, the term "transgene" denotes a construct which has been isolated by the "hand 25 of man" and transformed into the genome of a recipient organism. The dephosphorylation of transforming DNA may be carried out by any method capable of dephosphorylating DNA.

Dephosphorylation, the removal of a phosphate group, occurs by a process of "hydrolysis" in which the phosphate group is catalytically broken away ("lysed") from a parent molecule by the enzymatic addition of a water molecule to the parent molecule. 30 Dephosphorylation, as well as rephosphorylation, are part of essential processes in the energy-efficient functioning of all living cells. The dephosphorylation of a linear DNA molecule occurs by the removal of the highly-reactive terminal 5'-phosphate group. It is believed that

this removal of the highly-reactive terminal phosphate prevents the linear DNA molecule from spontaneously annealing to the 3'-hydroxyl group at the opposite end of the same molecule, or to terminal hydroxyl groups on other reactive DNA molecules in the same DNA composition.

Currently, the enzyme most widely used in molecular biology protocols for dephosphorylation is calf intestine alkaline phosphatase (see Maniatis *et al.*, Molecular Cloning: A Laboratory Manual; New York: Cold Spring Harbor Laboratory, pages 133-134, 1982; specifically incorporated herein by reference in its entirety). Calf alkaline phosphatase has advantages over other phosphatases in that its activity can be stopped by heating the reaction mixture to 68°C in the presence of an additional denaturing agent such as the detergent sodium dodecyl sulfate (SDS). Under these conditions, the calf intestine alkaline phosphatase loses its activity without denaturing the DNA in the reaction mixture.

A number of other known phosphatases have been isolated from *Aspergillus niger* (U.S. Patent No. 5,183,752; specifically incorporated herein by reference in its entirety). Reports have suggested the presence of as many as five acid phosphatase activities (*i.e.*, pH 2.5 to 5.0) in extracts of *A. niger* (Komano, 1975; Pathak and Sreenivasan, 1985); as well as several alkaline phosphatase activities from *A. niger* (functional pH optima from pH 8.5 to 10) (Rokosu and Uadia, 1980; Ramaswamy and Bheemeswar, 1976). Several distinct forms of phosphatase enzymes also have been isolated from cells of the yeast *Saccharomyces cerevisiae* (Attias and Bonnet, 1972), and have been found to function best in an environment having a pH optima between pH 8.0 and 8.5. Another known phosphatase is T₄ Polynucleotide kinase (commercially available from New England Biolabs), which will catalyze the removal of the 3' phosphate groups of a DNA molecule.

(ii) *Blunting of DNA Ends*

In addition to reducing transgene copy number by dephosphorylating DNA prior to transformation, it is specifically contemplated by the inventors that one may reduce transgene copy number by blunting the ends of the transforming DNA. It is believed that this will eliminate the "sticky-ends" of the DNA, and hence reduce the likelihood of concatamerization of transforming DNA prior to insertion in the target genome. The sticky ends may be present in a given DNA composition as a results of restriction with a staggered cutting restriction enzyme, or can occur as a result of random physical shearing of DNA molecules.

Methods contemplated by the inventors to be of particular utility in blunting DNA fragments are the use of DNA polymerases, single strand nucleases, or blunt cutting restriction

enzymes. Preferred DNA polymerases are *E. coli* DNA polymerase I, and more particularly, the Klenow fragment thereof. The Klenow fragment is a 76,000 dalton portion of the *E. coli* DNA Polymerase I enzyme containing the 5' → 3' polymerase and 3' → 5' exonuclease activities of the native enzyme, but lacking a smaller fragment (36,000 daltons) which exhibits only a 5' → 3' exonuclease activity. Both *E. coli* DNA Polymerase I and the Klenow fragment thereof are commercially available, for example, from New England Biolabs or Promega (e.g., Promega Cat. Nos. M2051, and M2201, respectively).

DNA polymerases such as the Klenow fragment will "fill in" the overhanging ends of a DNA segment to produce a blunt end. Some examples of other DNA polymerases deemed useful in this respect include T₄ DNA polymerase (Boehringer Mannheim), T₇ DNA polymerase (New England Biolabs), P_{W6} DNA polymerase (Boehringer Mannheim; Roche Molecular Systems and Perkin Elmer Corporation), and Vent and Deep Vent DNA polymerase (New England Biolabs). The current invention is not limited to the use of the aforementioned enzymes, however, as any DNA polymerase capable of producing blunt ends may potentially be used with the current invention.

As an alternative to filling in overhanging ends, it is specifically contemplated by the inventors that a single-strand DNA nuclease could be used to digest the overhang, thereby blunting the nucleic acid ends. Preferably, the nuclease will not digest double stranded DNA under the conditions at which the enzyme exhibits single strand nucleolytic activity. Some examples of single strand nucleases deemed especially useful with the current invention include S1 nuclease (commercially available from Pharmacia P-L Biochemicals, Piscataway, N.J.), RNase H, and mung bean nuclease, although any nuclease with similar activity can be used. Alternatively, it is contemplated that one could blunt DNA ends *via* restriction of the DNA with a blunt cutting restriction enzyme. Many blunt cutting restriction enzymes are known to those of skill in the art, for example, *EcoRV* and *SnaBI*.

General procedures for the above are known in the art and may be found in, for example, Berger and Kimmel, 1987, and Sambrook *et al.*, 1989, Fritsch, and Maniatis, *In Molecular Cloning: A Laboratory Manual*, Second edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989. specifically incorporated herein by reference in its entirety.

(iii) *DNA Concentration*

In accordance with the use of microprojectile bombardment with the current invention,

it is contemplated by the inventors that the proportion of single or low copy transformants obtained may be increased through the use of specific DNA concentrations for microprojectile preparation. Particularly, by lowering the effective concentration of transforming DNA, one may increase the proportion of single or low copy transformants. However, the DNA concentration used should not become too low, or the overall number of transformants will be decreased. Concentrations of transforming DNA which are deemed to be particularly useful for microprojectile bombardment with the current invention in the efficient production of single or low copy transformants are from about 1 ng to about 2000 ng of DNA per 1.8 mg of microprojectiles, or more preferably about 2.5 ng to about 1000 ng of DNA per 1.8 mg of microprojectiles, or even more preferably about 2.5 ng to about 100 ng of DNA per 1.8 mg of microprojectiles. It is specifically contemplated by the inventors, however, that any DNA concentration between about 1 ng and 1000 ng of DNA per 1.8g of microprojectiles will be of particular utility with the current invention, including about 5 ng, 10 ng, 20 ng, 50 ng, 100 ng, 150 ng, 250 ng, 500 ng, and about 750 ng of transforming DNA per 1.8 mg of microprojectiles.

The actual concentration chosen to use within the above ranges may depend on a number of factors. For example, if the transforming DNA has been blunted or dephosphorylated, it may be desirable to use a DNA concentration which is near the higher end of the preferred range, *i.e.*, about 750 ng to about 1000 ng of DNA per 1.8 mg of microprojectiles. It also is contemplated that the desired DNA concentration may be dependent upon the total molecular weight of the transforming DNA.

It will be understood to those of skill in the art that the above preferred DNA concentrations denote ratios of transforming DNA to microprojectiles, and that the actual amount of DNA used will vary according to the amount of microprojectiles used and the DNA composition used. It also will be understood that the above values denote the concentration of DNA which one desires to introduce into a host plant genome. Therefore, as an alternative to lowering the total amount of DNA used for transformation, it is contemplated that one could lower the effective concentration of transforming DNA by addition of one or more carrier DNAs. For example, one may include a second DNA sequence with the transforming DNA, thereby lowering the effective concentration of transforming DNA, without necessarily changing the overall quantity of DNA used for microprojectile preparation. Although potentially any DNA sequence could be used in this respect, DNA compositions comprising tRNA genes or repetitive sequences are deemed particularly useful in this respect. With regard

to tRNA as a carrier, it is believed that the tRNA will not integrate but will coprecipitate on the particles to "even out" the number of DNA molecules present, thereby limiting their interaction.

II. Transformation

There are many methods for transforming DNA segments into cells, but not all are suitable for delivering DNA to plant cells. Suitable methods for use with the current invention are believed to include virtually any method by which DNA can be introduced into a cell, such as by direct delivery of DNA such as by PEG-mediated transformation of protoplasts (Omirulleh *et al.*, 1993), by desiccation/inhibition-mediated DNA uptake (Potrykus *et al.*, 1985), by electroporation (U.S. Pat. No. 5,384,253, specifically incorporated herein by reference in its entirety), by agitation with silicon carbide fibers (Kaepller *et al.*, 1990; U.S. Pat. No. 5,302,523; and U.S. Pat. No. 5,464,765), and by acceleration of DNA coated particles (U.S. Pat. No. 5,550,318; U.S. Pat. No. 5,538,877; and U.S. Pat. No. 5,538,880; each specifically incorporated herein by reference in its entirety). Through the application of techniques such as these, certain cells from virtually any plant species may be stably transformed, and these cells developed into transgenic plants. In certain embodiments, acceleration methods are preferred and include, for example, microprojectile bombardment and the like.

(i) *Electroporation*

Where one wishes to introduce DNA by means of electroporation, it is contemplated that the method of Krzyzek *et al.* (U.S. Pat. No. 5,384,253, incorporated herein by reference in its entirety) will be particularly advantageous. In this method, certain cell wall-degrading enzymes, such as pectin-degrading enzymes, are employed to render the target recipient cells more susceptible to transformation by electroporation than untreated cells. Alternatively, recipient cells may be made more susceptible to transformation by mechanical wounding.

To effect transformation by electroporation, one may employ either friable tissues, such as a suspension culture of cells or embryogenic callus or alternatively one may transform immature embryos or other organized tissue directly. One would partially degrade the cell walls of the chosen cells by exposing them to pectin-degrading enzymes (pectolyases) or mechanically wounding in a controlled manner. Similarly, one may employ protoplasts of plant cells for transformation. Such cells would then be recipient to DNA transfer by electroporation, which may be carried out at this stage, and transformed cells then identified by

a suitable selection or screening protocol, dependent on the nature of the newly incorporated DNA.

(ii) *Microprojectile Bombardment*

A further advantageous method for delivering transforming DNA segments to plant cells is microprojectile bombardment (U.S. Pat. No. 5,550,318; U.S. Pat. No. 5,538,880; U.S. Pat. No. 5,610, 042; U.S. Pat. No. 5,015,580; and PCT Pat. Publication No. 94/09699; each of which is specifically incorporated herein by reference in its entirety). In this method, particles may be coated with nucleic acids and delivered into cells by a propelling force. Exemplary particles include those comprised of tungsten, gold, platinum, and the like. It is contemplated that in some instances DNA precipitation onto metal particles would not be necessary for DNA delivery to a recipient cell using microprojectile bombardment. However, it is contemplated that particles may contain DNA rather than be coated with DNA. Hence, it is proposed that DNA-coated particles may increase the level of DNA delivery via particle bombardment but are not, in and of themselves, necessary.

An illustrative embodiment of a method for delivering DNA into maize cells by acceleration is the Biolistics Particle Delivery System, which can be used to propel particles coated with DNA or cells through a screen, such as a stainless steel or Nytex screen, onto a filter surface covered with plant cells cultured in suspension. The screen disperses the particles so that they are not delivered to the recipient cells in large aggregates. It is believed that a screen intervening between the projectile apparatus and the cells to be bombarded reduces the size of projectiles aggregate and may contribute to a higher frequency of transformation by reducing the damage inflicted on the recipient cells by projectiles that are too large. Examples of species for which the Biolistics Particle Delivery System has been successfully used for transformation include monocot species such as maize, barley, wheat, rice, and sorghum, as well as various dicot species, including tobacco, soybean, cotton, sunflower, and tomato .

For the bombardment, cells in suspension are concentrated on filters or solid culture medium. Alternatively, immature embryos or other target cells may be arranged on solid culture medium. The cells to be bombarded are positioned at an appropriate distance below the macroprojectile stopping plate. If desired, one or more screens may be positioned between the acceleration device and the cells to be bombarded.

(iii) *Other Transformation Methods*

Transformation of plant protoplasts can be achieved using methods based on calcium phosphate precipitation, polyethylene glycol treatment, electroporation, and combinations of these treatments (see, e.g., Potrykus *et al.*, 1985; Lorz *et al.*, 1985; Omirulleh *et al.*, 1993; Fromm *et al.*, 1986; Uchimiya *et al.*, 1986; Callis *et al.*, 1987; Marcotte *et al.*, 1988). Application of these systems to different plant strains depends upon the ability to regenerate that particular plant strain from protoplasts. Illustrative methods for the regeneration of cereals from protoplasts are described (Fujimara *et al.*, 1985; Toriyama *et al.*, 1986; Yamada *et al.*, 1986; Abdullah *et al.*, 1986; Omirulleh *et al.*, and 1993 U.S. Pat. No. 5,508,184; each specifically incorporated herein by reference in its entirety).

To transform plant strains that cannot be successfully regenerated from protoplasts, other ways to introduce DNA into intact cells or tissues can be utilized. For example, regeneration of cereals from immature embryos or explants can be effected as described (Vasil, 1989).

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III. Optimization of Microprojectile Bombardment for Transformation Frequency

For microprojectile bombardment transformation in accordance with the current invention, both physical and biological parameters may be optimized. Physical factors are those that involve manipulating the DNA/microprojectile precipitate or those that affect the flight and velocity of either the macro- or microprojectiles. Biological factors include all steps involved in manipulation of cells before and immediately after bombardment, such as the osmotic adjustment of target cells to help alleviate the trauma associated with bombardment, the orientation of an immature embryo or other target tissue relative to the particle trajectory, and also the nature of the transforming DNA, such as linearized DNA or intact supercoiled plasmids. It is believed that pre-bombardment manipulations are especially important for successful transformation of immature embryos.

Accordingly, it is contemplated that one may wish to adjust various bombardment parameters in small scale studies to fully optimize the conditions. One may particularly wish to adjust physical parameters such as DNA concentration, gap distance, flight distance, tissue distance, and helium pressure. It is further contemplated that the grade of helium may effect transformation efficiency. For example, differences in transformation efficiencies may be witnessed between bombardments using industrial grade (99.99% pure) or ultra pure helium

(99.999% pure), although it is not currently clear which is more advantageous for use in bombardment. One also may optimize the trauma reduction factors (TRFs) by modifying conditions which influence the physiological state of the recipient cells and which may therefore influence transformation and integration efficiencies. For example, the osmotic state, 5 tissue hydration and the subculture stage or cell cycle of the recipient cells may be adjusted for optimum transformation.

Both physical and biological parameters for bombardment may be addressed for further optimization of ballistic transformation. Other physical factors include those that involve manipulating the DNA/microprojectile precipitate or those that affect the flight and velocity of 10 either the macro- or microprojectiles. Biological factors include all steps involved in manipulation of cells immediately before and after bombardment. The prebombardment culturing conditions, such as osmotic environment, the bombardment parameters, and the plasmid configuration have been adjusted to yield the maximum numbers of stable transformants.

15 (i) *Physical Parameters*

1. Gap Distance

The variable nest (macro holder) can be adjusted to vary the distance between the rupture disk and the macroprojectile, *i.e.*, the gap distance. This distance can be varied from 0 20 to 2 cm. The predicted effects of a shorter gap are an increase of velocity of both the macro- and microprojectiles, an increased shock wave (which leads to tissue splattering and increased tissue trauma), and deeper penetration of microprojectiles. Longer gap distances would have the opposite effects but may increase viability and therefore the total number of recovered stable transformants.

2. Flight Distance

25 The fixed nest (contained within the variable nest) can be varied between 0.5 and 2.25 cm in predetermined 0.5 cm increments by the placement of spacer rings to adjust the flight path traversed by the macroprojectile. Short flight paths allow for greater stability of the macroprojectile in flight but reduce the overall velocity of the microprojectiles. Increased 30 stability in flight increases, for example, the number of centered GUS foci. Greater flight distances (up to some point) increase velocity but also increase instability in flight. Based on observations, it is recommended that bombardments typically be done with a flight path length of about 1.0 cm to 1.5 cm.

3. Tissue Distance

Placement of tissue within the gun chamber can have significant effects on microprojectile penetration. Increasing the flight path of the microprojectiles will decrease velocity and trauma associated with the shock wave. A decrease in velocity also will result in shallower penetration of the microprojectiles.

5
4. Helium Pressure

By manipulation of the type and number of rupture disks, pressure can be varied between 400 and 2000 psi within the gas acceleration tube. Optimum pressure for stable transformation has been determined to be between 1000 and 1200 psi.

10
5. Coating of Microprojectiles.

For microprojectile bombardment, one will attach (*i.e.* "coat") DNA to the microprojectiles such that it is delivered to recipient cells in a form suitable for transformation thereof. In this respect, at least some of the transforming DNA must be available to the target cell for transformation to occur, while at the same time during delivery the DNA must be attached to the microprojectile. Therefore, availability of the transforming DNA from the microprojectile may comprise the physical reversal of interactions between transforming DNA and the microprojectile following delivery of the microprojectile to the target cell. This need not be the case, however, as availability to a target cell may occur as a result of breakage of unbound segments of DNA or of other molecules which comprise the physical attachment to the microprojectile. Availability may further occur as a result of breakage of bonds between the transforming DNA and other molecules, which are either directly or indirectly attached to the microprojectile. It is further contemplated that transformation of a target cell may occur by way of direct illegitimate or homology-dependent recombination between the transforming DNA and the genomic DNA of the recipient cell. Therefore, as used herein, a "coated" microprojectile will be one which is capable of being used to transform a target cell, in that the transforming DNA will be delivered to the target cell, yet will be accessible to the target cell such that transformation may occur.

Any technique for coating microprojectiles which allows for delivery of transforming DNA to the target cells may be used. Methods for coating microprojectiles which have been demonstrated to work well with the current invention have been specifically disclosed herein. DNA may be bound to microprojectile particles using alternative techniques, however. For example, particles may be coated with streptavidin and DNA end labeled with long chain thiol

cleavable biotinylated nucleotide chains. The DNA adheres to the particles due to the streptavidin-biotin interaction, but is released in the cell by reduction of the thiol linkage through reducing agents present in the cell.

5 Alternatively, particles may be prepared by functionalizing the surface of a gold oxide particle, providing free amine groups. DNA, having a strong negative charge, binds to the functionalized particles. Furthermore, charged particles may be deposited in controlled arrays on the surface of mylar flyer disks used in the PDS-1000 Biolistics device, thereby facilitating controlled distribution of particles delivered to target tissue.

10 As disclosed above, it further is proposed, that the concentration of DNA used to coat microprojectiles may influence the recovery of transformants containing a single copy of the transgene. For example, a lower concentration of DNA may not necessarily change the efficiency of the transformation, but may instead increase the proportion of single copy insertion events. Concentrations of DNA deemed particularly useful with the current invention are given above.

15 (ii) *Biological Parameters*

Culturing conditions and other factors can influence the physiological state of the target cells and may have profound effects on transformation and integration efficiencies. First, the act of bombardment could stimulate the production of ethylene which could lead to senescence of the tissue. The addition of antiethylene compounds could increase transformation 20 efficiencies. Second, it is proposed that certain points in the cell cycle may be more appropriate for integration of introduced DNA. Hence synchronization of cell cultures may enhance the frequency of production of transformants. For example, synchronization may be achieved using cold treatment, amino acid starvation, or other cell cycle-arresting agents. Third, the degree of tissue hydration also may contribute to the amount of trauma associated with 25 bombardment as well as the ability of the microprojectiles to penetrate cell walls.

The position and orientation of an embryo or other target tissue relative to the particle trajectory may also be important. For example, the PDS-1000 biolistics device does not produce a uniform spread of particles over the surface of a target petri dish. The velocity of particles in the center of the plate is higher than the particle velocity at further distances from 30 the center of the petri dish. Therefore, it is advantageous to situate target tissue on the petri dish such as to avoid the center of the dish, referred to by some as the "zone of death." Furthermore, orientation of the target tissue with regard to the trajectory of targets also can be

important. It is contemplated that it is desirable to orient the tissue most likely to regenerate a plant toward the particle stream. For example, the scutellum of an immature embryo comprises the cells of greatest embryogenic potential and therefore should be oriented toward the particle stream.

5 It also has been reported that slightly plasmolyzed yeast cells allow increased transformation efficiencies (Armaleo *et al.*, 1990). It was hypothesized that the altered osmotic state of the cells helped to reduce trauma associated with the penetration of the microprojectile. Additionally, the growth and cell cycle stage may be important with respect to transformation.

1. Osmotic Adjustment

10 It has been suggested that osmotic pre-treatment could potentially reduce bombardment associated injury as a result of the decreased turgor pressure of the plasmolyzed cell. In a previous study, the number of cells transiently expressing GUS increased following subculture into both fresh medium and osmotically adjusted medium (U.S. Pat. Appl. No. 08/113,561, filed August 25, 1993, specifically incorporated herein by reference in its entirety).
15 Pretreatment times of 90 minutes showed higher numbers of GUS expressing foci than shorter times. Cells incubated in 500 mOSM/kg medium for 90 minutes showed an approximately 3.5 fold increase in transient GUS foci than the control. Preferably, cells are precultured for 4-5 hours prior to bombardment on culture medium containing 12% sucrose. A second culture on 12% sucrose may be performed for 16-24 hours following bombardment. Alternatively, cells
20 are pretreated on 0.2M mannitol or 12% sucrose for 3-5 hours prior to bombardment. It is contemplated that pretreatment of cells with other osmotically active solutes for a period of 1-6 hours may also be desirable.

2. Plasmid Configuration

25 In some instances, it will be desirable to deliver DNA to maize cells that does not contain DNA sequences necessary for maintenance of the plasmid vector in the bacterial host, *e.g.*, *E. coli*, such as antibiotic resistance genes, including but not limited to ampicillin, kanamycin, and tetracycline resistance, and prokaryotic origins of DNA replication. In such case, a DNA fragment containing the transforming DNA may be purified prior to transformation. An exemplary method of purification is gel electrophoresis on a 1.2% low melting temperature agarose gel, followed by recovery from the agarose gel by melting gel slices in a 6-10 fold excess of Tris-EDTA buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 70°C-72°C); frozen and thawed (37°C); and the agarose pelleted by centrifugation. A Qiagen Q-100

column then may be used for purification of DNA. For efficient recovery of DNA, the flow rate of the column may be adjusted to 40 ml/hr.

Isolated DNA fragments can be recovered from agarose gels using a variety of electroelution techniques, enzyme digestion of the agarose, or binding of DNA to glass beads (e.g., Gene Clean™ or EluQuick™). In addition, HPLC and/or use of magnetic particles may be used to isolate DNA fragments. As an alternative to isolation of DNA fragments, a plasmid vector can be digested with a restriction enzyme and this DNA delivered to maize cells without prior purification of the expression cassette fragment.

10 **IV. Recipient Cells for Transformation**

Tissue culture requires media and controlled environments. "Media" refers to the numerous nutrient mixtures that are used to grow cells *in vitro*, that is, outside of the intact living organism. The medium usually is a suspension of various categories of ingredients (salts, amino acids, growth regulators, sugars, buffers) that are required for growth of most cell types. However, each specific cell type requires a specific range of ingredient proportions for growth, and an even more specific range of formulas for optimum growth. Rate of cell growth also will vary among cultures initiated with the array of media that permit growth of that cell type.

Nutrient media is prepared as a liquid, but this may be solidified by adding the liquid to materials capable of providing a solid support. Agar is most commonly used for this purpose. Bactoagar, Hazelton agar, Gelrite, and Gelgro are specific types of solid support that are suitable for growth of plant cells in tissue culture.

Some cell types will grow and divide either in liquid suspension or on solid media. As disclosed herein, maize cells will grow in suspension or on solid medium, but regeneration of plants from suspension cultures requires transfer from liquid to solid media at some point in development. The type and extent of differentiation of cells in culture will be affected not only by the type of media used and by the environment, for example, pH and light, but also by whether media is solid or liquid. Table 1 illustrates the composition of various media useful for creation of recipient cells and for plant regeneration.

Recipient cell targets include, but are not limited to, meristem cells, Type I, Type II, and Type III callus, immature embryos and gametic cells such as microspores, pollen, sperm and egg cells. It is contemplated that any cell from which a fertile transgenic plant may be

regenerated is useful as a recipient cell. Type I, Type II, and Type III callus may be initiated from tissue sources including, but not limited to, immature embryos, seedling apical meristems, microspores and the like. Those cells which are capable of proliferating as callus also are recipient cells for genetic transformation. The present invention provides techniques for transforming immature embryos and subsequent regeneration of fertile transgenic plants.

Transformation of immature embryos obviates the need for long term development of recipient cell cultures. Pollen, as well as its precursor cells, microspores, may be capable of functioning as recipient cells for genetic transformation, or as vectors to carry foreign DNA for incorporation during fertilization. Direct pollen transformation, or transformation of other germline cells such as microspores or megasporangia would obviate the need for cell culture.

Meristematic cells (*i.e.*, plant cells capable of continual cell division and characterized by an undifferentiated cytological appearance, normally found at growing points or tissues in plants such as root tips, stem apices, lateral buds, *etc.*) may represent another type of recipient plant cell. Because of their undifferentiated growth and capacity for organ differentiation and totipotency, a single transformed meristematic cell could be recovered as a whole transformed plant. In fact, it is proposed that embryogenic suspension cultures may be an *in vitro* meristematic cell system, retaining an ability for continued cell division in an undifferentiated state, controlled by the media environment.

Cultured plant cells that can serve as recipient cells for transforming with desired DNA segments may be any plant cells including corn cells, and more specifically, cells from *Zea mays* L. Somatic cells are of various types. Embryogenic cells are one example of somatic cells which may be induced to regenerate a plant through embryo formation. Non-embryogenic cells are those which typically will not respond in such a fashion. An example of non-embryogenic cells are certain Black Mexican Sweet (BMS) corn cells.

25 The development of embryogenic maize calli and suspension cultures useful in the context of the present invention, *e.g.*, as recipient cells for transformation, has been described in U.S. Pat. No. 5,134,074; and U.S. Pat. No. 5,489,520; each of which is incorporated herein by reference in its entirety.

Certain techniques may be used that enrich recipient cells within a cell population. For example, Type II callus development, followed by manual selection and culture of friable, embryogenic tissue, generally results in an enrichment of recipient cells for use in, microprojectile transformation. Suspension culturing, particularly using the media disclosed

herein, may improve the ratio of recipient to non-recipient cells in any given population. Manual selection techniques which can be employed to select recipient cells may include, e.g., assessing cell morphology and differentiation, or may use various physical or biological means. Cryopreservation is a possible method of selecting for recipient cells.

5 Manual selection of recipient cells, e.g., by selecting embryogenic cells from the surface of a Type II callus, is one means that may be used in an attempt to enrich for recipient cells prior to culturing (whether cultured on solid media or in suspension). The preferred cells may be those located at the surface of a cell cluster, and may further be identifiable by their lack of differentiation, their size and dense cytoplasm. The preferred cells will generally be those cells
10 which are less differentiated, or not yet committed to differentiation. Thus, one may wish to identify and select those cells which are cytoplasmically dense, relatively unvacuolated with a high nucleus to cytoplasm ratio (e.g., determined by cytological observations), small in size (e.g., 10-20 μm), and capable of sustained divisions and somatic proembryo formation.

15 It is proposed that other means for identifying such cells may also be employed. For example, through the use of dyes, such as Evan's blue, which are excluded by cells with relatively non-permeable membranes, such as embryogenic cells, and taken up by relatively differentiated cells such as root-like cells and snake cells (so-called due to their snake-like appearance).

20 Other possible means of identifying recipient cells include the use of isozyme markers of embryogenic cells, such as glutamate dehydrogenase, which can be detected by cytochemical stains (Fransz *et al.*, 1989). However, it is cautioned that the use of isozyme markers including glutamate dehydrogenase may lead to some degree of false positives from non-embryogenic cells such as rooty cells which nonetheless have a relatively high metabolic activity.

25 (i) *Culturing Cells to be Recipients for Transformation*

The inventors believe that the ability to prepare and cryopreserve cultures of maize cells is important to certain aspects of the present invention, in that it provides a means for reproducibly and successfully preparing cells for particle-mediated transformation. A variety of different types of media have been developed by the inventors and employed in carrying out various aspects of the invention. The following table, Table 1, sets forth the composition of the
30 media preferred by the inventors for carrying out these aspects of the invention.

Table 1

Tissue Culture Media Which are Used for Type II Callus Development, Development of Suspension Cultures and Regeneration of Plant Cells (Particularly Maize Cells)

| MEDIA NO. | BASAL MEDIUM | SUCROSE | pH | OTHER COMPONENTS** (Amount/L) |
|-----------|-----------------|---------|-----|--|
| 7 | MS* | 2% | 6.0 | .25 mg thiamine .5 mg BAP .5 mg NAA Bactoagar |
| 10 | MS | 2% | 6.0 | .25 mg thiamine 1 mg BAP 1 mg 2,4-D 400 mg L-proline Bactoagar |
| 19 | MS | 2% | 6.0 | .25 mg thiamine .25 mg BAP .25 mg NAA Bactoagar |
| 20 | MS | 3% | 6.0 | .25 mg 1 mg BAP 1 mg NAA Bactoagar |
| 52 | MS | 2% | 6.0 | .25 mg thiamine 1 mg 2,4-D 10^{-7} M ABA BACTOAGAR |

Table 1 - Continued

| MEDIA NO. | BASAL MEDIUM | SUCROSE | pH | OTHER COMPONENTS** (Amount/L) |
|-----------|-----------------|---------|-----|--|
| 101 | MS | 3% | 6.0 | MS vitamins 100 mg myo- inositol Bactoagar |
| 142 | MS | 6% | 6.0 | MS vitamins 5 mg BAP 0.186 mg NAA 0.175 mg IAA 0.403 mg 2IP Bactoagar |
| 157 | MS | 6% | 6.0 | MS vitamins 100 mg myo- inositol Bactoagar |
| 163 | MS | 3% | 6.0 | MS vitamins 3.3 mg dicamba 100 mg myo- inositol Bactoagar |
| 171 | MS | 3% | 6.0 | MS vitamins .25 mg 2,4-D 10 mg BAP 100 mg myo- inositol Bactoagar |

Table 1 - Continued

| MEDIA NO. | BASAL MEDIUM | SUCROSE | pH | OTHER COMPONENTS** (Amount/L) |
|-----------|-----------------|---------|-----|---|
| 173 | MS | 6% | 6.0 | MS vitamins 5 mg BAP .186 mg NAA .175 mg IAA .403 mg 2IP 10^{-7} M ABA 200 mg myo- inositol Bactoagar |
| 177 | MS | 3% | 6.0 | MS vitamins .25 mg 2,4-D 10 mg BAP 10^{-7} M ABA 100 mg myo- inositol Bactoagar |
| 185 | MS | - | 5.8 | 3 mg BAP .04 mg NAA RT vitamins 1.65 mg thiamine 1.38 g L-proline 20 g sorbitol Bactoagar |

Table 1 - Continued

| MEDIA NO. | BASAL MEDIUM | SUCROSE | pH | OTHER COMPONENTS** (Amount/L) |
|-----------|-----------------|---------|-----|--|
| 189 | MS | - | 5.8 | 3 mg BAP .04 mg NAA .5 mg niacin 800 mg L- asparagine 100 mg casamino acids 20 g sorbitol 1.4 g L-proline 100 mg myo- inositol Gelgro |
| 201 | N6 | 2% | 5.8 | N6 vitamins 2 mg L-glycine 1 mg 2,4-D 100 mg casein hydrolysate 2.9 g L-proline Gelgro |
| 205 | N6 | 2% | 5.8 | N6 vitamins 2 mg L-glycine .5 mg 2,4-D 100 mg casein hydrolysate 2.9 g L-proline Gelgro |

Table 1 - Continued

| MEDIA NO. | BASAL MEDIUM | SUCROSE | pH | OTHER COMPONENTS** (Amount/L) |
|------------------|-------------------------|----------------|-----------|--|
| 209 | N6 | 6% | 5.8 | N6 vitamins 2 mg L-glycine 100 mg casein hydrolysate 0.69 g L-proline Bactoagar |
| 210 | N6 | 3% | 5.5 | N6 vitamins 2 mg 2,4-D 250 mg Ca pantothenate 100 mg myo-inositol 790 mg L-asparagine 100 mg casein hydrolysate 1.4 g L-proline Hazelton agar**** 2 mg L-glycine |

Table 1 - Continued

| MEDIA NO. | BASAL MEDIUM | SUCROSE | pH | OTHER COMPONENTS** (Amount/L) |
|--|-----------------|---------|-----|--|
| 212 | N6 | 3% | 5.5 | N6 vitamins 2 mg L-glycine 2 mg 2,4-D 250 mg Ca pantothenate 100 mg myo-inositol 100 mg casein hydrolysate 1.4 g L-proline Hazelton agar**** |
| 227 | N6 | 2% | 5.8 | N6 vitamins 2 mg L-glycine 13.2 mg dicamba 100 mg casein hydrolysate 2.9 g L-proline Gelgro |
| 273 (also, 201V, 236S, 201D,2071, 2366, 201SV, 2377, and 201BV) | N6 | 2% | 5.8 | N6 vitamins 2 mg L-glycine 1 mg 2,4-D 16.9 mg AgNO ₃ 100 mg casein hydrolysate 2.9 g L-proline |

Table 1 - Continued

| MEDIA NO. | BASAL MEDIUM | SUCROSE | pH | OTHER COMPONENTS** (Amount/L) |
|-----------|-----------------|---------|-----|---|
| 279 | N6 | 2% | 5.8 | 3.3 mg dicamba 1 mg thiamine .5 mg niacin 800 mg L- asparagine 100 mg casein hydrolysate 100 mg myoinositol 1.4 g L-proline Gelgro**** |
| 288 | N6 | 3% | | 3.3 mg dicamba 1 mg thiamine .5 mg niacin .8 g L-asparagine 100 mg myo- inosital 1.4 g L-proline 100 mg casein hydrolysate 16.9 mg AgNO ₃ Gelgro |

Table 1 - Continued

| MEDIA NO. | BASAL MEDIUM | SUCROSE | pH | OTHER COMPONENTS** (Amount/L) |
|-----------|-----------------|---------|-----|--|
| 401 | MS | 3% | 6.0 | 3.73 mg Na ₂ EDTA .25 mg thiamine 1 mg 2,4-D 2 mg NAA 200 mg casein hydrolysate 500 mg K ₂ SO ₄ 400 mg KH ₂ PO ₄ 100 mg myo-inositol |
| 402 | MS | 3% | 6.0 | 3.73 mg Na ₂ EDTA .25 mg thiamine 1 mg 2,4-D 200 mg casein hydrolysate 2.9 g L-proline 500 mg K ₂ SO ₄ 400 mg KH ₂ PO ₄ 100 mg myo-inositol |

Table 1 - Continued

| MEDIA NO. | BASAL MEDIUM | SUCROSE | pH | OTHER COMPONENTS** (Amount/L) |
|-----------|-----------------------|---------|-----|--|
| 409 | MS | 3% | 6.0 | 3.73 mg Na ₂ EDTA .25 mg thiamine 9.9 mg dicamba 200 mg casein hydrolysate 2.9 g L-proline 500 mg K ₂ SO ₄ 400 mg KH ₂ PO ₄ 100 mg myo-inositol |
| 501 | Clark's Medium *** | 2% | 5.7 | |
| 607 | 1/2 x MS | 3% | 5.8 | 1 mg thiamine 1 mg niacin Gelrite |
| 615 | MS | 3% | 6.0 | MS vitamins 6 mg BAP 100 mg myo-inositol Bactoagar |
| 617 | 1/2 x MS | 1.5% | 6.0 | MS vitamins 50 mg myo-inositol Bactoagar |

Table 1 - Continued

| MEDIA NO. | BASAL MEDIUM | SUCROSE | pH | OTHER COMPONENTS** (Amount/L) |
|-----------|-----------------|---------|-----|--|
| 708 | N6 | 2% | 5.8 | N6 vitamins 2 mg L-glycine 1.5 mg 2,4-D 200 mg casein hydrolysate 0.69 g L-proline Gelrite |
| 721 | N6 | 2% | 5.8 | 3.3 mg dicamba 1 mg thiamine .5 mg niacin 800 mg L- asparagine 100 mg myo- inositol 100 mg casein hydrolysate 1.4 g L-proline 54.65 g mannitol Gelgro |

Table 1 - Continued

| MEDIA NO. | BASAL MEDIUM | SUCROSE | pH | OTHER COMPONENTS** (Amount/L) |
|------------------|-------------------------|----------------|-----------|--|
| 726 | N6 | 3% | 5.8 | 3.3 mg dicamba .5 mg niacin 1 mg thiamine 800 mg L- asparagine 100 mg myo- inositol 100 mg casein hydrolysate 1.4 g L-proline |
| 727 | N6 | 3% | 5.8 | N6 vitamins 2 mg L-glycine 9.9 mg dicamba 100 mg casein hydrolysate 2.9 g L-proline Gelgro |
| 728 | N6 | 3% | 5.8 | N6 vitamins 2 mg L-glycine 9.9 mg dicamba 16.9 mg AgNO ₃ 100 mg casein hydrolysate 2.9 g L-proline Gelgro |

Table 1 - Continued

| MEDIA NO. | BASAL MEDIUM | SUCROSE | pH | OTHER COMPONENTS** (Amount/L) |
|-----------|-----------------|---------|-----|---|
| 734 | N6 | 2% | 5.8 | N6 vitamins 2 mg L-glycine 1.5 mg 2,4-D 14 g Fe sequestreene (replaces Fe- EDTA) 200 mg casein hydrolyste 0.69 g L-proline Gelrite |
| 735 | N6 | 2% | 5.8 | 1 mg 2,4-D .5 mg niacin .91 g L-asparagine 100 mg myo- inositol 1 mg thiamine .5 g MES .75 g MgCl ₂ 100 mg casein hydrolysate 0.69 g L-proline Gelgro |

Table 1 - Continued

| MEDIA NO. | BASAL MEDIUM | SUCROSE | pH | OTHER COMPONENTS** (Amount/L) |
|-----------|-----------------|---------|-----|---|
| 2004 | N6 | 3% | 5.8 | 1 mg thiamine 0.5 mg niacin 3.3 mg dicamba 17 mg AgNO ₃ 1.4 g L-proline 0.8 g L-asparagine 100 mg casein hydrolysate 100 mg myo-inositol Gelrite |
| 2008 | N6 | 3% | 5.8 | 1 mg thiamine 0.5 mg niacin 3.3 mg dicamba 1.4 g L-proline 0.8 g L-asparagine Gelrite |

* Basic MS medium described in Murashige and Skoog (1962). This medium is typically modified by decreasing the NH₄NO₃ from 1.64 g/l to 1.55 g/l, and omitting the pyridoxine HCl, nicotinic acid, myo-inositol and glycine.

5 ** NAA = Napthal Acetic Acid

IAA = Indole Acetic Acid

2-IP = 2, isopentyl adenine

10 2,4-D = 2, 4-Dichlorophenoxyacetic Acid

BAP = 6-Benzyl aminopurine

ABA = abscisic acid

*** Basic medium described in Clark (1982)

**** These media may be made with or without solidifying agent.

A number of exemplary maize cultures which may be used for transformation have been developed and are disclosed in U.S. Pat. Appl. No. 08/113,561, filed August 25, 1993, which is specifically incorporated herein by reference.

(ii) *Media*

In certain embodiments, recipient cells are selected following growth in culture. Where employed, cultured cells may be grown either on solid supports or in the form of liquid suspensions. In either instance, nutrients may be provided to the cells in the form of media, and environmental conditions controlled. There are many types of tissue culture media comprised of various amino acids, salts, sugars, growth regulators and vitamins. Most of the media employed in the practice of the invention will have some similar components (see, Table 1), but may differ in the composition and proportions of their ingredients depending on the particular application envisioned. For example, various cell types usually grow in more than one type of media, but will exhibit different growth rates and different morphologies, depending on the growth media. In some media, cells survive but do not divide.

Various types of media suitable for culture of plant cells previously have been described. Examples of these media include, but are not limited to, the N6 medium described by Chu *et al.* (1975) and MS media (Murashige and Skoog, 1962). The inventors have discovered that media such as MS which have a high ammonia/nitrate ratio are counterproductive to the generation of recipient cells in that they promote loss of morphogenic capacity. N6 media, on the other hand, has a somewhat lower ammonia/nitrate ratio, and is contemplated to promote the generation of recipient cells by maintaining cells in a proembryonic state capable of sustained divisions.

(iii) *Maintenance*

The method of maintenance of cell cultures may contribute to their utility as sources of recipient cells for transformation. Manual selection of cells for transfer to fresh culture medium, frequency of transfer to fresh culture medium, composition of culture medium, and environment factors including, but not limited to, light quality and quantity and temperature are all important factors in maintaining callus and/or suspension cultures that are useful as sources of recipient cells. It is contemplated that alternating callus between different culture conditions may be beneficial in enriching for recipient cells within a culture. For example, it is proposed that cells may be cultured in suspension culture, but transferred to solid medium at regular intervals. After a period of growth on solid medium cells can be manually selected for return to

liquid culture medium. It is proposed that by repeating this sequence of transfers to fresh culture medium it is possible to enrich for recipient cells. It also is contemplated that passing cell cultures through a 1.9 mm sieve is useful in maintaining the friability of a callus or suspension culture and may be beneficial in enriching for transformable cells.

5 (iv) *Cryopreservation Methods*

Cryopreservation is important because it allows one to maintain and preserve a known transformable cell culture for future use, while eliminating the cumulative detrimental effects associated with extended culture periods.

10 Cell suspensions and callus were cryopreserved using modifications of methods previously reported (Finkle, 1985; Withers & King, 1979). The cryopreservation protocol comprised adding a pre-cooled (0°C) concentrated cryoprotectant mixture stepwise over a period of one to two hours to pre-cooled (0°C) cells. The mixture was maintained at 0°C throughout this period. The volume of added cryoprotectant was equal to the initial volume of the cell suspension (1:1 addition), and the final concentration of cryoprotectant additives was 15 10% dimethyl sulfoxide, 10% polyethylene glycol (6000 MW), 0.23 M proline and 0.23 M glucose. The mixture was allowed to equilibrate at 0°C for 30 minutes, during which time the cell suspension/ cryoprotectant mixture was divided into 1.5 ml aliquot (0.5 ml packed cell volume) in 2 ml polyethylene cryo-vials. The tubes were cooled at 0.5°C/minute to -8°C and held at this temperature for ice nucleation.

20 Once extracellular ice formation had been visually confirmed, the tubes were cooled at 0.5°C/minute from -8°C to -35°C. They were held at this temperature for 45 minutes (to insure uniform freeze-induced dehydration throughout the cell clusters). At this point, the cells had lost the majority of their osmotic volume (*i.e.*, there is little free water left in the cells), and they could be safely plunged into liquid nitrogen for storage. The paucity of free water remaining in 25 the cells in conjunction with the rapid cooling rates from -35°C to -196°C prevented large organized ice crystals from forming in the cells. The cells are stored in liquid nitrogen, which effectively immobilizes the cells and slows metabolic processes to the point where long-term storage should not be detrimental.

30 Thawing of the extracellular solution was accomplished by removing the cryo-tube from liquid nitrogen and swirling it in sterile 42°C water for approximately 2 minutes. The tube was removed from the heat immediately after the last ice crystals had melted to prevent heating the tissue. The cell suspension (still in the cryoprotectant mixture) was pipetted onto a filter,

resting on a layer of BMS cells (the feeder layer which provided a nurse effect during recovery). The cryoprotectant solution is removed by pipetting. Culture medium comprised a callus proliferation medium with increased osmotic strength. Dilution of the cryoprotectant occurred slowly as the solutes diffused away through the filter and nutrients diffused upward to 5 the recovering cells. Once subsequent growth of the thawed cells was noted, the growing tissue was transferred to fresh culture medium. If initiation of a suspension culture was desired, the cell clusters were transferred back into liquid suspension medium as soon as sufficient cell mass had been regained (usually within 1 to 2 weeks). Alternatively, cells were cultured on solid callus proliferation medium. After the culture was reestablished in liquid (within 1 to 2 10 additional weeks), it was used for transformation experiments. When desired, previously cryopreserved cultures may be frozen again for storage.

V. Identification of Transformed Cells Using Selection

It is believed that DNA is introduced into only a small percentage of target cells in any 15 one experiment. In order to provide an efficient system for identification of those cells receiving DNA and integrating it into their genomes one may employ a means for selecting those cells that are stably transformed. One exemplary embodiment of such a method is to introduce into the host cell, a marker gene which confers resistance to some normally inhibitory agent, such as an antibiotic or herbicide. Examples of antibiotics which may be used include 20 the aminoglycoside antibiotics neomycin, kanamycin and paromomycin, or the antibiotic hygromycin. Resistance to the aminoglycoside antibiotics is conferred by aminoglycoside phosphotransferase enzymes such as neomycin phosphotransferase II (NPT II) or NPT I, whereas resistance to hygromycin is conferred by hygromycin phosphotransferase.

Potentially transformed cells then are exposed to the selective agent. In the population 25 of surviving cells will be those cells where, generally, the resistance-conferring gene has been integrated and expressed at sufficient levels to permit cell survival. Cells may be tested further to confirm stable integration of the exogenous DNA. Using the techniques disclosed herein, greater than 40% of bombarded embryos may yield transformants.

One herbicide which has been suggested as a desirable selection agent is the broad 30 spectrum herbicide bialaphos. Bialaphos is a tripeptide antibiotic produced by *Streptomyces hygroscopicus* and is composed of phosphinothricin (PPT), an analogue of L-glutamic acid, and two L-alanine residues. Upon removal of the L-alanine residues by intracellular peptidases, the

PPT is released and is a potent inhibitor of glutamine synthetase (GS), a pivotal enzyme involved in ammonia assimilation and nitrogen metabolism (Ogawa *et al.*, 1973). Synthetic PPT, the active ingredient in the herbicide Liberty™ also is effective as a selection agent. Inhibition of GS in plants by PPT causes the rapid accumulation of ammonia and death of the plant cells.

The organism producing bialaphos and other species of the genus *Streptomyces* also synthesizes an enzyme phosphinothricin acetyl transferase (PAT) which is encoded by the *bar* gene in *Streptomyces hygroscopicus* and the *pat* gene in *Streptomyces viridochromogenes*. The use of the herbicide resistance gene encoding phosphinothricin acetyl transferase (PAT) is referred to in DE 3642 829 A, wherein the gene is isolated from *Streptomyces viridochromogenes*. In the bacterial source organism, this enzyme acetylates the free amino group of PPT preventing auto-toxicity (Thompson *et al.*, 1987). The *bar* gene has been cloned (Murakami *et al.*, 1986; Thompson *et al.*, 1987) and expressed in transgenic tobacco, tomato, potato (De Block, 1987) and *Brassica* (De Block, 1989) plants. In previous reports, some transgenic plants which expressed the resistance gene were completely resistant to commercial formulations of PPT and bialaphos in greenhouses.

Another example of a herbicide which is useful for selection of transformed cell lines in the practice of the invention is the broad spectrum herbicide glyphosate. Glyphosate inhibits the action of the enzyme EPSPS which is active in the aromatic amino acid biosynthetic pathway. Inhibition of this enzyme leads to starvation for the amino acids phenylalanine, tyrosine, and tryptophan and secondary metabolites derived therefrom. U.S. Pat. No. 4,535,060 describes the isolation of EPSPS mutations which confer glyphosate resistance on the *Salmonella typhimurium* gene for EPSPS, *aroA*. The EPSPS gene was cloned from *Zea mays* and mutations similar to those found in a glyphosate resistant *aroA* gene were introduced *in vitro*. Mutant genes encoding glyphosate resistant EPSPS enzymes are described in, for example, International Patent WO 97/4103. The best characterized mutant EPSPS gene conferring glyphosate resistance comprises amino acid changes at residues 102 and 106, although it is anticipated that other mutations will also be useful (PCT/WO97/4103).

It is further contemplated that the herbicide DALAPON, 2,2-dichloropropionic acid, may be useful for identification of transformed cells. The enzyme 2,2-dichloropropionic acid dehalogenase (*deh*) inactivates the herbicidal activity of 2,2-dichloropropionic acid and therefore confers herbicidal resistance on cells or plants expressing a gene encoding the

dehalogenase enzyme (Buchanan-Wollaston *et al.*, 1992; U.S. Pat. Appl. No. 08/113,561, filed August 25, 1993; U.S. Pat. No. 5,508,468; U.S. Pat. No. 5,508,468).

Alternatively, a gene encoding an anthranilate synthase gene which confers resistance to certain amino acid analogs, *e.g.*, 5-methyltryptophan or 6-methyl anthranilate, may be useful as a selectable marker gene. The use of an anthranilate synthase gene as a selectable marker was described in U.S. Pat. No. 5,508,468; and U.S. Pat. Application 08/604,789.

VI. DNA Sequences

It is well known in the art that virtually any DNA composition may be introduced with any given transformation technique to ultimately produce fertile transgenic plants. The construction of vectors which may be employed in conjunction with the present invention will be known to those of skill of the art in light of the present disclosure (see for example, Sambrook *et al.*, 1989; Gelvin *et al.*, 1990). The techniques of the current invention are thus not limited to any particular DNA sequences. For example, DNA segments in the form of vectors and plasmids, or linear DNA fragments, in some instances containing only the DNA element to be expressed in the plant, and the like, may be employed.

In certain embodiments, it is contemplated that one may wish to employ replication-competent viral vectors in monocot transformation. Such vectors include, for example, wheat dwarf virus (WDV) "shuttle" vectors, such as pW1-11 and PW1-GUS (Ugaki *et al.*, 1991). These vectors are capable of autonomous replication in maize cells as well as *E. coli*, and as such may provide increased sensitivity for detecting DNA delivered to transgenic cells. A replicating vector also may be useful for delivery of genes flanked by DNA sequences from transposable elements such as Ac, Ds, or Mu. It has been proposed (Laufs *et al.*, 1990) that transposition of these elements within the maize genome requires DNA replication. It also is contemplated that transposable elements would be useful for introducing DNA fragments lacking elements necessary for selection and maintenance of the plasmid vector in bacteria, *e.g.*, antibiotic resistance genes and origins of DNA replication. It also is proposed that use of a transposable element such as Ac, Ds, or Mu would actively promote integration of the desired DNA and hence increase the frequency of stably transformed cells.

It further is contemplated that one may wish to co-transform plants or plant cells with 2 or more vectors. Co-transformation may be achieved using a vector containing the marker and another gene or genes of interest. Alternatively, different vectors, *e.g.*, plasmids, may contain

the different genes of interest, and the plasmids may be concurrently delivered to the recipient cells. Using this method, the assumption is made that a certain percentage of cells in which the marker has been introduced, also have received the other gene(s) of interest. Thus, not all cells selected by means of the marker, will express the other genes of interest which had been presented to the cells concurrently.

Vectors, plasmids, cosmids, YACs (yeast artificial chromosomes), BACs (bacterial artificial chromosomes) and other DNA segments for use in transforming plant cells will, of course, generally comprise the cDNA, gene or genes which one desires to introduce into the cells. These DNA constructs can further include structures such as promoters, enhancers, polylinkers, or even regulatory genes as desired. The DNA segment or gene chosen for cellular introduction will often encode a protein which will be expressed in the resultant recombinant cells, such as will result in a screenable or selectable trait and/or which will impart an improved phenotype to the regenerated plant. However, this may not always be the case, and the present invention also encompasses transgenic plants incorporating non-expressed transgenes. Preferred components likely to be included with vectors used in the current invention are as follows.

(i) *Regulatory Elements*

Preferred constructs generally will include a plant promoter. Useful promoters include those that are inducible, viral, synthetic, constitutive as described (Poszkowski *et al.*, 1989; Odell *et al.*, 1985), temporally regulated, spatially regulated, and spatio-temporally regulated (Chau *et al.*, 1989). A promoter is selected for its ability to direct the transformed plant cell's or transgenic plant's transcriptional activity to the coding region. Promoters can be near-constitutive, such as the CaMV 35S promoter (Odell *et al.*, 1985), histone, CaMV 19S (Lawton *et al.*, 1987), nos (Ebert *et al.*, 1987), Adh (Walker *et al.*, 1987), sucrose synthase (Yang & Russell, 1990), α -tubulin, actin (Wang *et al.*, 1992), cab (Sullivan *et al.*, 1989), PEPCase (Hudspeth & Grula, 1989) and R gene complex-associated promoters (Chandler *et al.*, 1989). Where the promoter is a near-constitutive promoter, increases in polypeptide expression generally are found in a variety of transformed plant tissues (*e.g.*, callus, leaf, seed and root).

Alternatively, expression of transgenes can be directed to specific plant tissues by using vectors containing a tissue-specific promoter. An exemplary tissue-specific promoter is the lectin promoter (Vodkin *et al.*, 1983; Lindstrom *et al.*, 1990.). Other exemplary tissue-specific promoters are corn sucrose synthetase 1 (Yang *et al.*, 1990), corn alcohol dehydrogenase 1

(Vogel *et al.*, 1989), corn light harvesting complex (Simpson, 1986), corn heat shock protein (Odell *et al.*, 1985), pea small subunit RuBP carboxylase (Poulsen *et al.*, 1986; Cashmore *et al.*, 1983), Ti plasmid mannopine synthase (Langridge *et al.*, 1989), Ti plasmid nopaline synthase (Langridge *et al.*, 1989), petunia chalcone isomerase (Van Tunen *et al.*, 1988), bean glycine rich protein 1 (Keller *et al.*, 1989), truncated CaMV 35s (Odell *et al.*, 1985), Potato patatin promoters (Wenzler *et al.*, 1989), maize zein and globulin-1 promoters. Other tissue specific promoters including root cell promoters (Conkling *et al.*, 1990) and tissue specific enhancers (Fromm *et al.*, 1989) also are contemplated to be particularly useful, as are inducible promoters such as ABA- and turgor-inducible promoters.

Another type of element which can regulate gene expression is the DNA sequence between the transcription initiation site and the start of the coding sequence, termed the untranslated leader sequence. The leader sequence can influence gene expression and compilations of leader sequences have been made to predict optimum or sub-optimum sequences and generate "consensus" and preferred leader sequences (Joshi, 1987). Preferred leader sequences are contemplated to include those which include sequences predicted to direct optimum expression of the attached gene, *i.e.*, to include a preferred consensus leader sequence which may increase or maintain mRNA stability and prevent inappropriate initiation of translation. The choice of such sequences will be known to those of skill in the art in light of the present disclosure. Sequences that are derived from genes that are highly expressed in plants, and in maize in particular, will be most preferred.

Transcription enhancers or duplications of enhancers could be used to increase expression. These enhancers often are found 5' to the start of transcription in a promoter that functions in eukaryotic cells, but can often be inserted in the forward or reverse orientation 5' or 3' to the coding sequence. Examples of enhancers include elements from the CaMV 35S promoter, octopine synthase genes (Ellis *et al.*, 1987), the rice actin gene, and promoters from non-plant eukaryotes (*e.g.* yeast; Ma *et al.*, 1988).

Specifically contemplated for use in accordance with the present invention are vectors which include the ocs enhancer element. This element was first identified as a 16 bp palindromic enhancer from the octopine synthase (ocs) gene of *Agrobacterium* (Ellis *et al.*, 1987), and is present in at least 10 other promoters (Bouchez *et al.*, 1989). It is proposed that the use of an enhancer element, such as the ocs element and particularly multiple copies of the

element, may be used to increase the level of transcription from adjacent promoters when applied in the context of monocot transformation.

It is contemplated that introduction of large DNA sequences comprising more than one gene may be desirable. Introduction of such sequences may be facilitated by use of bacterial or 5 yeast artificial chromosomes (BACs or YACs, respectively), or even plant artificial chromosomes. For example, the use of BACs for *Agrobacterium*-mediated transformation was disclosed by Hamilton *et al.* (1996).

Ultimately, the most desirable DNA segments for introduction into a monocot genome may be homologous genes or gene families which encode a desired trait (for example, increased 10 yield per acre), and which are introduced under the control of novel promoters or enhancers, etc., or perhaps even homologous or tissue specific (*e.g.*, root-, collar/sheath-, whorl-, stalk-, earshank-, kernel- or leaf-specific) promoters or control elements. Indeed, it is envisioned that a particular use of the present invention may be the production of transformants comprising a 15 transgene which is targeted in a tissue-specific manner. For example, insect resistant genes may be expressed specifically in the whorl and collar/sheath tissues which are targets for the first and second broods, respectively, of European Corn Borer (ECB). Likewise, genes encoding proteins with particular activity against rootworm may be targeted directly to root tissues.

Vectors for use in tissue-specific targeting of gene expression in transgenic plants 20 typically will include tissue-specific promoters and may also include other tissue-specific control elements such as enhancer sequences. Promoters which direct specific or enhanced expression in certain plant tissues will be known to those of skill in the art in light of the present disclosure.

It also is contemplated that tissue specific expression may be functionally accomplished 25 by introducing a constitutively expressed gene (all tissues) in combination with an antisense gene that is expressed only in those tissues where the gene product is not desired. For example, a gene coding for the crystal toxin protein from *B. thuringiensis* (Bt) may be introduced such that it is expressed in all tissues using the 35S promoter from Cauliflower Mosaic Virus. Alternatively, a rice actin promoter or a histone promoter from a dicot or 30 monocot species could also be used for constitutive expression of a gene. Furthermore, it is contemplated that promoters combining elements from more than one promoter may be useful. For example, U.S. Pat. No. 5,491,288 discloses combining a Cauliflower Mosaic Virus

promoter with a histone promoter. Therefore, expression of an antisense transcript of the Bt gene in a maize kernel, using for example a zein promoter, would prevent accumulation of the Bt protein in seed. Hence the protein encoded by the introduced gene would be present in all tissues except the kernel.

5 Alternatively, one may wish to obtain novel tissue-specific promoter sequences for use in accordance with the present invention. To achieve this, one may first isolate cDNA clones from the tissue concerned and identify those clones which are expressed specifically in that tissue, for example, using Northern blotting. Ideally, one would like to identify a gene that is not present in a high copy number, but which gene product is relatively abundant in specific
10 tissues. The promoter and control elements of corresponding genomic clones may then be localized using the techniques of molecular biology known to those of skill in the art.

15 It is contemplated that expression of some genes in transgenic plants will be desired only under specified conditions. For example, it is proposed that expression of certain genes that confer resistance to environmental stress factors such as drought will be desired only under actual stress conditions. It further is contemplated that expression of such genes throughout a
20 plants development may have detrimental effects. It is known that a large number of genes exist that respond to the environment. For example, expression of some genes such as rbcS, encoding the small subunit of ribulose bisphosphate carboxylase, is regulated by light as mediated through phytochrome. Other genes are induced by secondary stimuli. For example, synthesis of abscisic acid (ABA) is induced by certain environmental factors, including but not limited to water stress. A number of genes have been shown to be induced by ABA (Skriver and Mundy, 1990). It also is anticipated that expression of genes conferring resistance to insect predation would be desired only under conditions of actual insect infestation. Therefore, for some desired traits, inducible expression of genes in transgenic plants will be desired.

25 It is proposed that, in some embodiments of the present invention, expression of a gene in a transgenic plant will be desired only in a certain time period during the development of the plant. Developmental timing frequently is correlated with tissue specific gene expression. For example, expression of zein storage proteins is initiated in the endosperm about 15 days after pollination.

30 It also is contemplated that it may be useful to target DNA itself within a cell. For example, it may be useful to target introduced DNA to the nucleus as this may increase the frequency of transformation. Within the nucleus itself it would be useful to target a gene in

order to achieve site specific integration. For example, it would be useful to have a gene introduced through transformation replace an existing gene in the cell.

(ii) *Terminators*

Constructs will typically include the gene of interest along with a 3' end DNA sequence that acts as a signal to terminate transcription and allow for the poly-adenylation of the resultant mRNA. The most preferred 3' elements are contemplated to be those from the nopaline synthase gene of *Agrobacterium tumefaciens* (nos 3' end) (Bevan *et al.*, 1983), the terminator for the T7 transcript from the octopine synthase gene of *Agrobacterium tumefaciens*, and the 3' end of the protease inhibitor I or II genes from potato or tomato. Regulatory elements such as Adh intron 1 (Callis *et al.*, 1987), sucrose synthase intron (Vasil *et al.*, 1989) or TMV omega element (Gallie, *et al.*, 1989), may further be included where desired.

(iii) *Transit or signal peptides.*

Sequences that are joined to the coding sequence of the resistance gene, which are removed post-translationally from the initial translation product and which facilitate the transport of the protein into or through intracellular or extracellular membranes are termed transit (usually into vacuoles, vesicles, plastids and other intracellular organelles) and signal sequences (usually to the outside of the cellular membrane). By facilitating the transport of the protein into compartments inside and outside the cell these sequences may increase the accumulation of gene product protecting them from proteolytic degradation. These sequences also allow for additional mRNA sequences from highly expressed genes to be attached to the coding sequence of the genes. Since mRNA being translated by ribosomes is more stable than naked mRNA, the presence of translatable mRNA in front of the gene may increase the overall stability of the mRNA transcript from the gene and thereby increase synthesis of the gene product. Since transit and signal sequences are usually post-translationally removed from the initial translation product, the use of these sequences allows for the addition of extra translated sequences that may not appear on the final polypeptide. It is further contemplated that targeting of certain proteins may be desirable in order to enhance the stability of the protein (U.S. Pat. No. 5,545,818, incorporated herein by reference in its entirety).

Additionally, vectors may be constructed and employed in the intracellular targeting of a specific gene product within the cells of a transgenic plant or in directing a protein to the extracellular environment. This generally will be achieved by joining a DNA sequence encoding a transit or signal peptide sequence to the coding sequence of a particular gene. The

resultant transit, or signal, peptide will transport the protein to a particular intracellular, or extracellular destination, respectively, and will then be post-translationally removed. Transit or signal peptides act by facilitating the transport of proteins through intracellular membranes, e.g., vacuole, vesicle, plastid and mitochondrial membranes, whereas signal peptides direct 5 proteins through the extracellular membrane.

A particular example of such a use concerns the direction of a protein conferring herbicide resistance, such as the mutant EPSPS protein, to a particular organelle such as the chloroplast rather than to the cytoplasm. This is exemplified by the use of the rbcS transit peptide, the chloroplast transit peptide described in European Pat. No. 0189707, or the 10 optimized transit peptide described in U.S. Pat. No. 5,510,471, which confers plastid-specific targeting of proteins. In addition, it may be desirable to target certain genes responsible for male sterility to the mitochondria, or to target certain genes for resistance to phytopathogenic organisms to the extracellular spaces, or to target proteins to the vacuole. A further use concerns the direction of enzymes involved in amino acid biosynthesis or oil synthesis to the 15 plastid. Such enzymes include dihydridipicolinic acid synthase which may contribute to increasing lysine content of a feed.

(iv) *Marker Genes*

In order to improve the ability to identify transformants, one may employ a selectable or screenable marker gene as, or in addition to, the expressible gene of interest. "Marker genes" 20 are genes that impart a distinct phenotype to cells expressing the marker gene and thus allow such transformed cells to be distinguished from cells that do not have the marker. Such genes may encode either a selectable or screenable marker, depending on whether the marker confers a trait which one can "select" for by chemical means, i.e., through the use of a selective agent (e.g., a herbicide, antibiotic, or the like), or whether it is simply a trait that one can identify 25 through observation or testing, i.e., by "screening" (e.g., the R-locus trait). Of course, many examples of suitable marker genes are known to the art and can be employed in the practice of the invention.

Included within the terms selectable or screenable marker genes also are genes which 30 encode a "secretable marker" whose secretion can be detected as a means of identifying or selecting for transformed cells. Examples include markers which encode a secretable antigen that can be identified by antibody interaction, or even secretable enzymes which can be detected by their catalytic activity. Secretable proteins fall into a number of classes, including

small, diffusible proteins detectable, *e.g.*, by ELISA; small active enzymes detectable in extracellular solution (*e.g.*, α -amylase, β -lactamase, phosphinothricin acetyltransferase); and proteins that are inserted or trapped in the cell wall (*e.g.*, proteins that include a leader sequence such as that found in the expression unit of extensin or tobacco PR-S).

5 With regard to selectable secretable markers, the use of a gene that encodes a protein that becomes sequestered in the cell wall, and which protein includes a unique epitope is considered to be particularly advantageous. Such a secreted antigen marker would ideally employ an epitope sequence that would provide low background in plant tissue, a promoter-leader sequence that would impart efficient expression and targeting across the plasma membrane, and would produce protein that is bound in the cell wall and yet accessible to antibodies. A normally secreted wall protein modified to include a unique epitope would satisfy all such requirements.

10 One example of a protein suitable for modification in this manner is extensin, or hydroxyproline rich glycoprotein (HPRG). The use of maize HPRG (Steifel *et al.*, 1990) is preferred, as this molecule is well characterized in terms of molecular biology, expression and protein structure. However, any one of a variety of extensins and/or glycine-rich wall proteins 15 (Keller *et al.*, 1989) could be modified by the addition of an antigenic site to create a screenable marker.

20 One exemplary embodiment of a secretable screenable marker concerns the use of a maize sequence encoding the wall protein HPRG, modified to include a 15 residue epitope from the pro-region of murine interleukin-1- β (IL-1- β). However, virtually any detectable epitope may be employed in such embodiments, as selected from the extremely wide variety of antigen:antibody combinations known to those of skill in the art. The unique extracellular 25 epitope, whether derived from IL-1 β or any other protein or epitopic substance, can then be straightforwardly detected using antibody labeling in conjunction with chromogenic or fluorescent adjuncts.

1. Selectable Markers

30 Many selectable marker genes may be used in connection with the present invention including, but not limited to, a *neo* gene (Potrykus *et al.*, 1985) which codes for kanamycin resistance and can be selected for using kanamycin, G418, paromomycin, etc.; a *bar* gene which confers bialaphos resistance; a mutant EPSP synthase protein (Hinchee *et al.*, 1988) conferring glyphosate resistance; a nitrilase gene such as *bxn* from *Klebsiella ozaenae* which

confers resistance to bromoxynil (Stalker *et al.*, 1988); a mutant acetolactate synthase gene (ALS) which confers resistance to imidazolinone, sulfonylurea or other ALS inhibiting chemicals (European Patent Application 154,204, 1985); a methotrexate resistant DHFR gene (Thillet *et al.*, 1988), a dalapon dehalogenase gene that confers resistance to the herbicide dalapon; or a mutated anthranilate synthase gene that confers resistance to 5-methyl tryptophan. Where a mutant EPSP synthase gene is employed, additional benefit may be realized through the incorporation of a suitable chloroplast transit peptide, CTP (U.S. Pat. No. 5,188,642).

An illustrative embodiment of selectable marker genes capable of being used in systems to select transformants are the genes that encode the enzyme phosphinothricin acetyltransferase, such as the *bar* gene from *Streptomyces hygroscopicus* or the *pat* gene from *Streptomyces viridochromogenes*. The enzyme phosphinothricin acetyl transferase (PAT) inactivates the active ingredient in the herbicide bialaphos, phosphinothricin (PPT). PPT inhibits glutamine synthetase, (Murakami *et al.*, 1986; Twell *et al.*, 1989) causing rapid accumulation of ammonia and cell death.

Where one desires to employ a bialaphos resistance gene in the practice of the invention, the inventors have discovered that particularly useful genes for this purpose are the *bar* or *pat* genes obtainable from species of *Streptomyces* (*e.g.*, ATCC No. 21,705). The cloning of the *bar* gene has been described (Murakami *et al.*, 1986; Thompson *et al.*, 1987) as has the use of the *bar* gene in the context of plants other than monocots (De Block *et al.*, 1987; De Block *et al.*, 1989).

20. 2. Screenable Markers

Screenable markers that may be employed include a β -glucuronidase or *uidA* gene (GUS) which encodes an enzyme for which various chromogenic substrates are known; an R-locus gene, which encodes a product that regulates the production of anthocyanin pigments (red color) in plant tissues (Dellaporta *et al.*, 1988); a β -lactamase gene (Sutcliffe, 1978), which encodes an enzyme for which various chromogenic substrates are known (*e.g.*, PADAC, a chromogenic cephalosporin); a *xylE* gene (Zukowsky *et al.*, 1983) which encodes a catechol dioxygenase that can convert chromogenic catechols; an α -amylase gene (Ikuta *et al.*, 1990); a tyrosinase gene (Katz *et al.*, 1983) which encodes an enzyme capable of oxidizing tyrosine to DOPA and dopaquinone which in turn condenses to form the easily-detectable compound melanin; a β -galactosidase gene, which encodes an enzyme for which there are chromogenic substrates; a luciferase (*lux*) gene (Ow *et al.*, 1986), which allows for bioluminescence detection; an aequorin

gene (Prasher *et al.*, 1985) which may be employed in calcium-sensitive bioluminescence detection; or a gene encoding for green fluorescent protein (Sheen *et al.*, 1995).

Genes from the maize R gene complex are contemplated to be particularly useful as screenable markers. The R gene complex in maize encodes a protein that acts to regulate the production of anthocyanin pigments in most seed and plant tissue. Maize strains can have one, or as many as four, R alleles which combine to regulate pigmentation in a developmental and tissue specific manner. Thus, an R gene introduced into such cells will cause the expression of a red pigment and, if stably incorporated, can be visually scored as a red sector. If a maize line carries dominant alleles for genes encoding for the enzymatic intermediates in the anthocyanin biosynthetic pathway (C2, A1, A2, Bz1 and Bz2), but carries a recessive allele at the R locus, transformation of any cell from that line with R will result in red pigment formation. Exemplary lines include Wisconsin 22 which contains the rg-Stadler allele and TR112, a K55 derivative which is r-g, b, Pl. Alternatively, any genotype of maize can be utilized if the C1 and R alleles are introduced together.

The inventors further propose that R gene regulatory regions may be employed in chimeric constructs in order to provide mechanisms for controlling the expression of chimeric genes. More diversity of phenotypic expression is known at the R locus than at any other locus (Coe *et al.*, 1988). It is contemplated that regulatory regions obtained from regions 5' to the structural R gene would be valuable in directing the expression of genes for, *e.g.*, insect resistance, herbicide tolerance or other protein coding regions. For the purposes of the present invention, it is believed that any of the various R gene family members may be successfully employed (*e.g.*, P, S, Lc, *etc.*). However, the most preferred will generally be Sn (particularly Sn:bol3). Sn is a dominant member of the R gene complex and is functionally similar to the R and B loci in that Sn controls the tissue specific deposition of anthocyanin pigments in certain seedling and plant cells, therefore, its phenotype is similar to R.

A further screenable marker contemplated for use in the present invention is firefly luciferase, encoded by the *lux* gene. The presence of the *lux* gene in transformed cells may be detected using, for example, X-ray film, scintillation counting, fluorescent spectrophotometry, low-light video cameras, photon counting cameras or multiwell luminometry. It also is envisioned that this system may be developed for populational screening for bioluminescence, such as on tissue culture plates, or even for whole plant screening. The gene which encodes green fluorescent protein is contemplated as a particularly useful reporter gene (Sheen *et al.*, 1995).

Expression of green fluorescent protein may be visualized in a cell or plant as fluorescence following illumination by particular wavelengths of light.

(v) *Transgenes for Modification of Monocots*

A particularly important advance of the present invention is that it provides methods and compositions for the efficient transformation of plant cells with genes in addition to, or other than, marker genes. Such transgenes often will be genes that direct the expression of a particular protein or polypeptide product, but they may also be non-expressible DNA segments, e.g., transposons such as Ds that do not direct their own transposition. As used herein, an "expressible gene" is any gene that is capable of being transcribed into RNA (e.g., mRNA, antisense RNA, etc.) or translated into a protein, expressed as a trait of interest, or the like, etc., and is not limited to selectable, screenable or non-selectable marker genes. The invention also contemplates that, where both an expressible gene that is not necessarily a marker gene is employed in combination with a marker gene, one may employ the separate genes on either the same or different DNA segments for transformation. In the latter case, the different vectors are delivered concurrently to recipient cells to maximize cotransformation.

The choice of the particular DNA segments to be delivered to the recipient cells often will depend on the purpose of the transformation. One of the major purposes of transformation of crop plants is to add some commercially desirable, agronomically important traits to the plant. Such traits include, but are not limited to, herbicide resistance or tolerance; insect resistance or tolerance; disease resistance or tolerance (viral, bacterial, fungal, nematode); stress tolerance and/or resistance, as exemplified by resistance or tolerance to drought, heat, chilling, freezing, excessive moisture, salt stress; oxidative stress; increased yields; food content and makeup; physical appearance; male sterility; drydown; standability; prolificacy; starch quantity and quality; oil quantity and quality; protein quality and quantity; amino acid composition; and the like. One may desire to incorporate one or more genes conferring any such desirable trait or traits, such as, for example, a gene or genes encoding herbicide resistance.

In certain embodiments, the present invention contemplates the transformation of a recipient cell with more than one advantageous transgene. Two or more transgenes can be supplied in a single transformation event using either distinct transgene-encoding vectors, or using a single vector incorporating two or more gene coding sequences. For example, plasmids bearing the *bar* and *aroA* expression units in either convergent, divergent, or colinear orientation, are considered to be particularly useful. Further preferred combinations are those of an insect

resistance gene, such as a Bt gene, along with a protease inhibitor gene such as *pinII*, or the use of *bar* in combination with either of the above genes. Of course, any two or more transgenes of any description, such as those conferring herbicide, insect, disease (viral, bacterial, fungal, nematode) or drought resistance, male sterility, drydown, standability, prolificacy, starch properties, oil quantity and quality, or those increasing yield or nutritional quality may be employed as desired.

5 1. Herbicide Resistance

The genes encoding phosphinothrin acetyltransferase (*bar* and *pat*), glyphosate tolerant EPSP synthase genes, the glyphosate degradative enzyme gene *gox* encoding glyphosate oxidoreductase, *deh* (encoding a dehalogenase enzyme that inactivates dalapon), herbicide resistant (e.g., sulfonylurea and imidazolinone) acetolactate synthase, and *bxn* genes (encoding a nitrilase enzyme that degrades bromoxynil) are good examples of herbicide resistant genes for use in transformation. The *bar* and *pat* genes code for an enzyme, phosphinothrin acetyltransferase (PAT), which inactivates the herbicide phosphinothrin and prevents this compound from inhibiting glutamine synthetase enzymes. The enzyme 5-enolpyruvylshikimate 3-phosphate synthase (EPSP Synthase), is normally inhibited by the herbicide N-(phosphonomethyl)glycine (glyphosate). However, genes are known that encode glyphosate-resistant EPSP synthase enzymes. These genes are particularly contemplated for use in monocot transformation. The *deh* gene encodes the enzyme dalapon dehalogenase and confers resistance to the herbicide dalapon. The *bxn* gene codes for a specific nitrilase enzyme that converts bromoxynil to a non-herbicidal degradation product.

10 2. Insect Resistance

Potential insect resistance genes that can be introduced include *Bacillus thuringiensis* crystal toxin genes or Bt genes (Watrud *et al.*, 1985). Bt genes may provide resistance to lepidopteran or coleopteran pests such as European Corn Borer (ECB). Preferred Bt toxin genes for use in such embodiments include the *CryIA(b)* and *CryIA(c)* genes. Endotoxin genes from other species of *B. thuringiensis* which affect insect growth or development may also be employed in this regard.

The poor expression of prokaryotic Bt toxin genes in plants is a well-documented phenomenon, and the use of different promoters, fusion proteins, and leader sequences has not led to significant increases in Bt protein expression (Vaeck *et al.*, 1987; Barton *et al.*, 1987). It is therefore contemplated that the most advantageous Bt genes for use in the transformation protocols disclosed herein will be those in which the coding sequence has been modified to effect

increased expression in plants, and more particularly, those in which maize preferred codons have been used. Means for preparing synthetic genes are well known in the art and are disclosed in, for example, U.S. Pat. No. 5,380,831, specifically incorporated herein by reference in its entirety. Examples of such modified Bt toxin genes include a synthetic Bt *CryIA(b)* gene (Perlak *et al.*, 5 1991), and the synthetic *CryIA(c)* gene termed 1800b (PCT Application WO 95/06128). Some examples of other Bt toxin genes known to those skill in the art are given in Table 2 below.

Table 2
***Bacillus thuringiensis* δ-Endotoxin Genes^a**

| New Nomenclature | Old Nomenclature | GenBank Accession # |
|------------------|------------------|---------------------|
| Cry1Aa | CryIA(a) | M11250 |
| Cry1Ab | CryIA(b) | M13898 |
| Cry1Ac | CryIA(c) | M11068 |
| Cry1Ad | CryIA(d) | M73250 |
| Cry1Ae | CryIA(e) | M65252 |
| Cry1Ba | CryIB | X06711 |
| Cry1Bb | ET5 | L32020 |
| Cry1Bc | PEG5 | Z46442 |
| Cry1Bd | CryE1 | U70726 |
| Cry1Ca | CryIC | X07518 |
| Cry1Cb | CryIC(b) | M97880 |
| Cry1Da | CryID | X54160 |
| Cry1Db | PrtB | Z22511 |
| Cry1Ea | CryIE | X53985 |
| Cry1Eb | CryIE(b) | M73253 |
| Cry1Fa | CryIF | M63897 |
| Cry1Fb | PrtD | Z22512 |
| Cry1Ga | PrtA | Z22510 |
| Cry1Gb | CryH2 | U70725 |
| Cry1Ha | PrtC | Z22513 |
| Cry1Hb | | U35780 |
| Cry1Ia | CryV | X62821 |

Table 2 - Continued

| New Nomenclature | Old Nomenclature | GenBank Accession # |
|------------------|------------------|---------------------|
| Cry1Ib | CryV | U07642 |
| Cry1Ja | ET4 | L32019 |
| Cry1Jb | ET1 | U31527 |
| Cry1K | | U28801 |
| Cry2Aa | CryIIA | M31738 |
| Cry2Ab | CryIIB | M23724 |
| Cry2Ac | CryIIC | X57252 |
| Cry3A | CryIIIA | M22472 |
| Cry3Ba | CryIIIB | X17123 |
| Cry3Bb | CryIIIB2 | M89794 |
| Cry3C | CryIID | X59797 |
| Cry4A | CryIVA | Y00423 |
| Cry4B | CryIVB | X07423 |
| Cry5Aa | CryVA(a) | L07025 |
| Cry5Ab | CryVA(b) | L07026 |
| Cry6A | CryVIA | L07022 |
| Cry6B | CryVIB | L07024 |
| Cry7Aa | CryIIIC | M64478 |
| Cry7Ab | CryIIICb | U04367 |
| Cry8A | CryIIIE | U04364 |
| Cry8B | CryIIIG | U04365 |
| Cry8C | CryIIIF | U04366 |
| Cry9A | CryIG | X58120 |
| Cry9B | CryIX | X75019 |
| Cry9C | CryIH | Z37527 |
| Cry10A | CryIVC | M12662 |
| Cry11A | CryIVD | M31737 |
| Cry11B | Jeg80 | X86902 |
| Cry12A | CryVB | L07027 |
| Cry13A | CryVC | L07023 |

Table 2 - Continued

| New Nomenclature | Old Nomenclature | GenBank Accession # |
|------------------|------------------|---------------------|
| Cry14A | CryVD | U13955 |
| Cry15A | 34kDa | M76442 |
| Cry16A | cbm71 | X94146 |
| Cry17A | cbm71 | X99478 |
| Cry18A | CryBP1 | X99049 |
| Cry19A | Jeg65 | Y08920 |
| Cyt1Aa | CytA | X03182 |
| Cyt1Ab | CytM | X98793 |
| Cyt2A | CytB | Z14147 |
| Cyt2B | CytB | U52043 |

^aAdapted from: http://epunix.biols.susx.ac.uk/Home/Neil_Crickmore/Bt/index.html

Protease inhibitors also may provide insect resistance (Johnson *et al.*, 1989), and will thus have utility in plant transformation. The use of a protease inhibitor II gene, *pinII*, from tomato or potato is envisioned to be particularly useful. Even more advantageous is the use of a *pinII* gene in combination with a Bt toxin gene, the combined effect of which has been discovered by the present inventors to produce synergistic insecticidal activity. Other genes which encode inhibitors of the insects' digestive system, or those that encode enzymes or co-factors that facilitate the production of inhibitors, also may be useful. This group may be exemplified by oryzacystatin and amylase inhibitors such as those from wheat and barley.

Also, genes encoding lectins may confer additional or alternative insecticide properties. Lectins (originally termed phytohemagglutinins) are multivalent carbohydrate-binding proteins which have the ability to agglutinate red blood cells from a range of species. Lectins have been identified recently as insecticidal agents with activity against weevils, ECB and rootworm (Murdock *et al.*, 1990; Czapla & Lang, 1990). Lectin genes contemplated to be useful include, for example, barley and wheat germ agglutinin (WGA) and rice lectins (Gatehouse *et al.*, 1984), with WGA being preferred.

Genes controlling the production of large or small polypeptides active against insects when introduced into the insect pests, such as, e.g., lytic peptides, peptide hormones and toxins

and venoms, form another aspect of the invention. For example, it is contemplated that the expression of juvenile hormone esterase, directed towards specific insect pests, may also result in insecticidal activity, or perhaps cause cessation of metamorphosis (Hammock *et al.*, 1990).

Transgenic maize expressing genes which encode enzymes that affect the integrity of the insect cuticle form yet another aspect of the invention. Such genes include those encoding, *e.g.*, chitinase, proteases, lipases and also genes for the production of nikkomycin, a compound that inhibits chitin synthesis, the introduction of any of which is contemplated to produce insect resistant maize plants. Genes that code for activities that affect insect molting, such as those affecting the production of ecdysteroid UDP-glucosyl transferase, also fall within the scope of the useful transgenes of the present invention.

Genes that code for enzymes that facilitate the production of compounds that reduce the nutritional quality of the host plant to insect pests also are encompassed by the present invention. It may be possible, for instance, to confer insecticidal activity on a plant by altering its sterol composition. Sterols are obtained by insects from their diet and are used for hormone synthesis and membrane stability. Therefore alterations in plant sterol composition by expression of novel genes, *e.g.*, those that directly promote the production of undesirable sterols or those that convert desirable sterols into undesirable forms, could have a negative effect on insect growth and/or development and hence endow the plant with insecticidal activity. Lipoxygenases are naturally occurring plant enzymes that have been shown to exhibit anti-nutritional effects on insects and to reduce the nutritional quality of their diet. Therefore, further embodiments of the invention concern transgenic plants with enhanced lipoxygenase activity which may be resistant to insect feeding.

Tripsacum dactyloides is a species of grass that is resistant to certain insects, including corn root worm. It is anticipated that genes encoding proteins that are toxic to insects or are involved in the biosynthesis of compounds toxic to insects will be isolated from *Tripsacum* and that these novel genes will be useful in conferring resistance to insects. It is known that the basis of insect resistance in *Tripsacum* is genetic, because said resistance has been transferred to *Zea mays* via sexual crosses (Branson and Guss, 1972).

Further genes encoding proteins characterized as having potential insecticidal activity may also be used as transgenes in accordance herewith. Such genes include, for example, the cowpea trypsin inhibitor (CpTI; Hilder *et al.*, 1987) which may be used as a rootworm deterrent; genes encoding avermectin (*Avermectin and Abamectin*, Campbell, W.C., Ed., 1989; Ikeda *et al.*, 1987)

which may prove particularly useful as a corn rootworm deterrent; ribosome inactivating protein genes; and even genes that regulate plant structures. Transgenic maize including anti-insect antibody genes and genes that code for enzymes that can convert a non-toxic insecticide (pro-insecticide) applied to the outside of the plant into an insecticide inside the plant also are contemplated.

5 3. Environment or Stress Resistance

Improvement of corn's ability to tolerate various environmental stresses such as, but not limited to, drought, excess moisture, chilling, freezing, high temperature, salt, and oxidative stress, can also be effected through expression of novel genes. It is proposed that benefits may 10 be realized in terms of increased resistance to freezing temperatures through the introduction of an "antifreeze" protein such as that of the Winter Flounder (Cutler *et al.*, 1989) or synthetic gene derivatives thereof. Improved chilling tolerance may also be conferred through increased expression of glycerol-3-phosphate acetyltransferase in chloroplasts (Wolter *et al.*, 1992). Resistance to oxidative stress (often exacerbated by conditions such as chilling temperatures in 15 combination with high light intensities) can be conferred by expression of superoxide dismutase (Gupta *et al.*, 1993), and may be improved by glutathione reductase (Bowler *et al.*, 1992). Such strategies may allow for tolerance to freezing in newly emerged fields as well as extending later maturity higher yielding varieties to earlier relative maturity zones.

It is contemplated that the expression of novel genes that favorably effect plant water 20 content, total water potential, osmotic potential, and turgor will enhance the ability of the plant to tolerate drought. As used herein, the terms "drought resistance" and "drought tolerance" are used to refer to a plants increased resistance or tolerance to stress induced by a reduction in water availability, as compared to normal circumstances, and the ability of the plant to function and survive in lower-water environments. In this aspect of the invention it is proposed, for 25 example, that the expression of genes encoding for the biosynthesis of osmotically-active solutes, such as polyol compounds, may impart protection against drought. Within this class are genes encoding for mannitol-L-phosphate dehydrogenase (Lee and Saier, 1982) and trehalose-6-phosphate synthase (Kaasen *et al.*, 1992). Through the subsequent action of native phosphatases in the cell or by the introduction and coexpression of a specific phosphatase, these 30 introduced genes will result in the accumulation of either mannitol or trehalose, respectively, both of which have been well documented as protective compounds able to mitigate the effects of stress. Mannitol accumulation in transgenic tobacco has been verified and preliminary

results indicate that plants expressing high levels of this metabolite are able to tolerate an applied osmotic stress (Tarczynski *et al.*, 1992, 1993).

Similarly, the efficacy of other metabolites in protecting either enzyme function (*e.g.*, alanopine or propionic acid) or membrane integrity (*e.g.*, alanopine) has been documented (Loomis *et al.*, 1989), and therefore expression of genes encoding for the biosynthesis of these compounds might confer drought resistance in a manner similar to or complimentary to mannitol. Other examples of naturally occurring metabolites that are osmotically active and/or provide some direct protective effect during drought and/or desiccation include fructose, erythritol (Coxson *et al.*, 1992), sorbitol, dulcitol (Karsten *et al.*, 1992), glucosylglycerol (Reed *et al.*, 1984; Erdmann *et al.*, 1992), sucrose, stachyose (Koster and Leopold, 1988; Blackman *et al.*, 1992), raffinose (Bernal-Lugo and Leopold, 1992), proline (Rensburg *et al.*, 1993), glycine betaine, ononitol and pinitol (Vernon and Bohnert, 1992). Continued canopy growth and increased reproductive fitness during times of stress will be augmented by introduction and expression of genes such as those controlling the osmotically active compounds discussed above and other such compounds. Currently preferred genes which promote the synthesis of an osmotically active polyol compound are genes which encode the enzymes mannitol-1-phosphate dehydrogenase, trehalose-6-phosphate synthase and myoinositol 0-methyltransferase.

It is contemplated that the expression of specific proteins may also increase drought tolerance. Three classes of Late Embryogenic Proteins have been assigned based on structural similarities (see Dure *et al.*, 1989). All three classes of LEAs have been demonstrated in maturing (*i.e.* desiccating) seeds. Within these 3 types of LEA proteins, the Type-II (dehydron-type) have generally been implicated in drought and/or desiccation tolerance in vegetative plant parts (*i.e.* Mundy and Chua, 1988; Piatkowski *et al.*, 1990; Yamaguchi-Shinozaki *et al.*, 1992). Recently, expression of a Type-III LEA (HVA-1) in tobacco was found to influence plant height, maturity and drought tolerance (Fitzpatrick, 1993). In rice, expression of the HVA-1 gene influenced tolerance to water deficit and salinity (Xu *et al.*, 1996). Expression of structural genes from all three LEA groups may therefore confer drought tolerance. Other types of proteins induced during water stress include thiol proteases, aldolases and transmembrane transporters (Guerrero *et al.*, 1990), which may confer various protective and/or repair-type functions during drought stress. It also is contemplated that genes that effect lipid biosynthesis

and hence membrane composition might also be useful in conferring drought resistance on the plant.

Many of these genes for improving drought resistance have complementary modes of action. Thus, it is envisaged that combinations of these genes might have additive and/or synergistic effects in improving drought resistance in corn. Many of these genes also improve freezing tolerance (or resistance); the physical stresses incurred during freezing and drought are similar in nature and may be mitigated in similar fashion. Benefit may be conferred via constitutive expression of these genes, but the preferred means of expressing these novel genes may be through the use of a turgor-induced promoter (such as the promoters for the turgor-induced genes described in Guerrero *et al.*, 1990 and Shagan *et al.*, 1993 which are incorporated herein by reference). Spatial and temporal expression patterns of these genes may enable corn to better withstand stress.

It is proposed that expression of genes that are involved with specific morphological traits that allow for increased water extractions from drying soil would be of benefit. For example, introduction and expression of genes that alter root characteristics may enhance water uptake. It also is contemplated that expression of genes that enhance reproductive fitness during times of stress would be of significant value. For example, expression of genes that improve the synchrony of pollen shed and receptiveness of the female flower parts, *i.e.*, silks, would be of benefit. In addition it is proposed that expression of genes that minimize kernel abortion during times of stress would increase the amount of grain to be harvested and hence be of value.

Given the overall role of water in determining yield, it is contemplated that enabling corn to utilize water more efficiently, through the introduction and expression of novel genes, will improve overall performance even when soil water availability is not limiting. By introducing genes that improve the ability of corn to maximize water usage across a full range of stresses relating to water availability, yield stability or consistency of yield performance may be realized.

4. Disease Resistance

It is proposed that increased resistance to diseases may be realized through introduction of genes into monocotyledonous plants such as maize. It is possible to produce resistance to diseases caused by viruses, bacteria, fungi and nematodes. It also is contemplated that control of mycotoxin producing organisms may be realized through expression of introduced genes.

Resistance to viruses may be produced through expression of novel genes. For example, it has been demonstrated that expression of a viral coat protein in a transgenic plant can impart resistance to infection of the plant by that virus and perhaps other closely related viruses (Cuozzo *et al.*, 1988, Hemenway *et al.*, 1988, Abel *et al.*, 1986). It is contemplated that expression of antisense genes targeted at essential viral functions may impart resistance to said virus. For example, an antisense gene targeted at the gene responsible for replication of viral nucleic acid may inhibit replication and lead to resistance to the virus. It is believed that interference with other viral functions through the use of antisense genes may also increase resistance to viruses. Further, it is proposed that it may be possible to achieve resistance to viruses through other approaches, including, but not limited to the use of satellite viruses.

It is proposed that increased resistance to diseases caused by bacteria and fungi may be realized through introduction of novel genes. It is contemplated that genes encoding so-called "peptide antibiotics," pathogenesis related (PR) proteins, toxin resistance, and proteins affecting host-pathogen interactions such as morphological characteristics will be useful. Peptide antibiotics are polypeptide sequences which are inhibitory to growth of bacteria and other microorganisms. For example, the classes of peptides referred to as cecropins and magainins inhibit growth of many species of bacteria and fungi. It is proposed that expression of PR proteins in monocotyledonous plants such as maize may be useful in conferring resistance to bacterial disease. These genes are induced following pathogen attack on a host plant and have been divided into at least five classes of proteins (Bol, Linthorst, and Cornelissen, 1990). Included amongst the PR proteins are β -1,3-glucanases, chitinases, and osmotin and other proteins that are believed to function in plant resistance to disease organisms. Other genes have been identified that have antifungal properties, *e.g.*, UDA (stinging nettle lectin) and hevein (Broekaert *et al.*, 1989; Barkai-Golan *et al.*, 1978). It is known that certain plant diseases are caused by the production of phytotoxins. It is proposed that resistance to these diseases would be achieved through expression of a novel gene that encodes an enzyme capable of degrading or otherwise inactivating the phytotoxin. It also is contemplated that expression of novel genes that alter the interactions between the host plant and pathogen may be useful in reducing the ability of the disease organism to invade the tissues of the host plant, *e.g.*, an increase in the waxiness of the leaf cuticle or other morphological characteristics.

Plant parasitic nematodes are a cause of disease in many plants, including maize. It is proposed that it would be possible to make the corn plant resistant to these organisms through

the expression of novel genes. It is anticipated that control of nematode infestations would be accomplished by altering the ability of the nematode to recognize or attach to a host plant and/or enabling the plant to produce nematicidal compounds, including but not limited to proteins.

5 5. Mycotoxin Reduction/Elimination

Production of mycotoxins, including aflatoxin and fumonisin, by fungi associated with monocotyledonous plants such as maize is a significant factor in rendering the grain not useful. These fungal organisms do not cause disease symptoms and/or interfere with the growth of the plant, but they produce chemicals (mycotoxins) that are toxic to animals. It is contemplated that inhibition of the growth of these fungi would reduce the synthesis of these toxic substances and therefore reduce grain losses due to mycotoxin contamination. It also is proposed that it may be possible to introduce novel genes into monocotyledonous plants such as maize that would inhibit synthesis of the mycotoxin without interfering with fungal growth. Further, it is contemplated that expression of a novel gene which encodes an enzyme capable of rendering the mycotoxin nontoxic would be useful in order to achieve reduced mycotoxin contamination of grain. The result of any of the above mechanisms would be a reduced presence of mycotoxins on grain.

6. Grain Composition or Quality

Genes may be introduced into monocotyledonous plants, particularly commercially important cereals such as maize, to improve the grain for which the cereal is primarily grown. A wide range of novel transgenic plants produced in this manner may be envisioned depending on the particular end use of the grain.

The largest use of maize grain is for feed or food. Introduction of genes that alter the composition of the grain may greatly enhance the feed or food value. The primary components of maize grain are starch, protein, and oil. Each of these primary components of maize grain may be improved by altering its level or composition. Several examples may be mentioned for illustrative purposes, but in no way provide an exhaustive list of possibilities.

The protein of cereal grains including maize is suboptimal for feed and food purposes especially when fed to pigs, poultry, and humans. The protein is deficient in several amino acids that are essential in the diet of these species, requiring the addition of supplements to the grain. Limiting essential amino acids may include lysine, methionine, tryptophan, threonine, valine, arginine, and histidine. Some amino acids become limiting only after corn is

supplemented with other inputs for feed formulations. For example, when corn is supplemented with soybean meal to meet lysine requirements methionine becomes limiting. The levels of these essential amino acids in seeds and grain may be elevated by mechanisms which include, but are not limited to, the introduction of genes to increase the biosynthesis of 5 the amino acids, decrease the degradation of the amino acids, increase the storage of the amino acids in proteins, or increase transport of the amino acids to the seeds or grain.

One mechanism for increasing the biosynthesis of the amino acids is to introduce genes that deregulate the amino acid biosynthetic pathways such that the plant can no longer adequately control the levels that are produced. This may be done by deregulating or bypassing 10 steps in the amino acid biosynthetic pathway which are normally regulated by levels of the amino acid end product of the pathway. Examples include the introduction of genes that encode deregulated versions of the enzymes aspartokinase or dihydrolipidic acid (DHDP)- synthase for increasing lysine and threonine production, and anthranilate synthase for increasing tryptophan production. Reduction of the catabolism of the amino acids may be 15 accomplished by introduction of DNA sequences that reduce or eliminate the expression of genes encoding enzymes that catalyze steps in the catabolic pathways such as the enzyme lysine-ketoglutarate reductase.

The protein composition of the grain may be altered to improve the balance of amino acids in a variety of ways including elevating expression of native proteins, decreasing 20 expression of those with poor composition, changing the composition of native proteins, or introducing genes encoding entirely new proteins possessing superior composition. Examples may include the introduction of DNA that decreases the expression of members of the zein family of storage proteins. This DNA may encode ribozymes or antisense sequences directed to impairing expression of zein proteins or expression of regulators of zein expression such as 25 the opaque-2 gene product. It also is proposed that the protein composition of the grain may be modified through the phenomenon of co-suppression, *i.e.*, inhibition of expression of an endogenous gene through the expression of an identical structural gene or gene fragment introduced through transformation (Goring *et al.*, 1991). Additionally, the introduced DNA may encode enzymes which degrade zeins. The decreases in zein expression that are achieved 30 may be accompanied by increases in proteins with more desirable amino acid composition or increases in other major seed constituents such as starch. Alternatively, a chimeric gene may be introduced that comprises a coding sequence for a native protein of adequate amino acid

composition such as for one of the globulin proteins or 10 kD zein of maize and a promoter or other regulatory sequence designed to elevate expression of said protein. The coding sequence of said gene may include additional or replacement codons for essential amino acids. Further, a coding sequence obtained from another species, or, a partially or completely synthetic sequence 5 encoding a completely unique peptide sequence designed to enhance the amino acid composition of the seed may be employed.

The introduction of genes that alter the oil content of the grain may be of value. Increases in oil content may result in increases in metabolizable-energy-content and density of the seeds for use in feed and food. The introduced genes may encode enzymes that remove or 10 reduce rate-limitations or regulated steps in fatty acid or lipid biosynthesis. Such genes may include, but are not limited to, those that encode acetyl-CoA carboxylase, ACP-acyltransferase, β -ketoacyl-ACP synthase, plus other well known fatty acid biosynthetic activities. Other possibilities are genes that encode proteins that do not possess enzymatic activity such as acyl carrier protein. Genes may be introduced that alter the balance of fatty acids present in the oil 15 providing a more healthful or nutritive feedstuff. The introduced DNA also may encode sequences that block expression of enzymes involved in fatty acid biosynthesis, altering the proportions of fatty acids present in the grain such as described below.

Genes may be introduced that enhance the nutritive value of the starch component of the grain, for example by increasing the degree of branching, resulting in improved utilization of 20 the starch in cows by delaying its metabolism.

Besides affecting the major constituents of the grain, genes may be introduced that affect a variety of other nutritive, processing, or other quality aspects of the grain as used for feed or food. For example, pigmentation of the grain may be increased or decreased. Enhancement and stability of yellow pigmentation is desirable in some animal feeds and may 25 be achieved by introduction of genes that result in enhanced production of xanthophylls and carotenes by eliminating rate-limiting steps in their production. Such genes may encode altered forms of the enzymes phytoene synthase, phytoene desaturase, or lycopene synthase. Alternatively, unpigmented white corn is desirable for production of many food products and may be produced by the introduction of DNA which blocks or eliminates steps in pigment 30 production pathways.

Feed or food comprising primarily maize or other cereal grains possesses insufficient quantities of vitamins and must be supplemented to provide adequate nutritive value.

Introduction of genes that enhance vitamin biosynthesis in seeds may be envisioned including, for example, vitamins A, E, B₁₂, choline, and the like. Maize grain also does not possess sufficient mineral content for optimal nutritive value. Genes that affect the accumulation or availability of compounds containing phosphorus, sulfur, calcium, manganese, zinc, and iron among others would be valuable. An example may be the introduction of a gene that reduced phytic acid production or encoded the enzyme phytase which enhances phytic acid breakdown. These genes would increase levels of available phosphate in the diet, reducing the need for supplementation with mineral phosphate.

Numerous other examples of improvement of maize or other cereals for feed and food purposes might be described. The improvements may not even necessarily involve the grain, but may, for example, improve the value of the corn for silage. Introduction of DNA to accomplish this might include sequences that alter lignin production such as those that result in the "brown midrib" phenotype associated with superior feed value for cattle.

In addition to direct improvements in feed or food value, genes also may be introduced which improve the processing of corn and improve the value of the products resulting from the processing. The primary method of processing corn is via wetmilling. Maize may be improved through the expression of novel genes that increase the efficiency and reduce the cost of processing such as by decreasing steeping time.

Improving the value of wetmilling products may include altering the quantity or quality of starch, oil, corn gluten meal, or the components of corn gluten feed. Elevation of starch may be achieved through the identification and elimination of rate limiting steps in starch biosynthesis or by decreasing levels of the other components of the grain resulting in proportional increases in starch. An example of the former may be the introduction of genes encoding ADP-glucose pyrophosphorylase enzymes with altered regulatory activity or which are expressed at higher level. Examples of the latter may include selective inhibitors of, for example, protein or oil biosynthesis expressed during later stages of kernel development.

The properties of starch may be beneficially altered by changing the ratio of amylose to amylopectin, the size of the starch molecules, or their branching pattern. Through these changes a broad range of properties may be modified which include, but are not limited to, changes in gelatinization temperature, heat of gelatinization, clarity of films and pastes, rheological properties, and the like. To accomplish these changes in properties, genes that encode granule-bound or soluble starch synthase activity or branching enzyme activity may be

introduced alone or combination. DNA such as antisense constructs may also be used to decrease levels of endogenous activity of these enzymes. The introduced genes or constructs may possess regulatory sequences that time their expression to specific intervals in starch biosynthesis and starch granule development. Furthermore, it may be worthwhile to introduce 5 and express genes that result in the *in vivo* derivatization, or other modification, of the glucose moieties of the starch molecule. The covalent attachment of any molecule may be envisioned, limited only by the existence of enzymes that catalyze the derivatizations and the accessibility of appropriate substrates in the starch granule. Examples of important derivations may include the addition of functional groups such as amines, carboxyls, or phosphate groups which provide sites for subsequent *in vitro* derivatizations or affect starch properties through the introduction 10 of ionic charges. Examples of other modifications may include direct changes of the glucose units such as loss of hydroxyl groups or their oxidation to aldehyde or carboxyl groups.

Oil is another product of wetmilling of corn, the value of which may be improved by introduction and expression of genes. The quantity of oil that can be extracted by wetmilling 15 may be elevated by approaches as described for feed and food above. Oil properties also may be altered to improve its performance in the production and use of cooking oil, shortenings, lubricants or other oil-derived products or improvement of its health attributes when used in the food-related applications. Novel fatty acids also may be synthesized which upon extraction can serve as starting materials for chemical syntheses. The changes in oil properties may be achieved by altering the type, level, or lipid arrangement of the fatty acids present in the oil. 20 This in turn may be accomplished by the addition of genes that encode enzymes that catalyze the synthesis of novel fatty acids and the lipids possessing them or by increasing levels of native fatty acids while possibly reducing levels of precursors. Alternatively, DNA sequences may be introduced which slow or block steps in fatty acid biosynthesis resulting in the increase 25 in precursor fatty acid intermediates. Genes that might be added include desaturases, epoxidases, hydratases, dehydratases, and other enzymes that catalyze reactions involving fatty acid intermediates. Representative examples of catalytic steps that might be blocked include the desaturations from stearic to oleic acid and oleic to linolenic acid resulting in the respective accumulations of stearic and oleic acids. Another example is the blockage of elongation steps 30 resulting in the accumulation of C₈ to C₁₂ saturated fatty acids.

Improvements in the other major corn wetmilling products, corn gluten meal and corn gluten feed, may also be achieved by the introduction of genes to obtain novel corn plants.

Representative possibilities include but are not limited to those described above for improvement of food and feed value.

In addition, it may further be considered that the corn plant be used for the production or manufacturing of useful biological compounds that were either not produced at all, or not 5 produced at the same level, in the corn plant previously. The novel corn plants producing these compounds are made possible by the introduction and expression of genes by corn transformation methods. The vast array of possibilities include but are not limited to any biological compound which is presently produced by any organism such as proteins, nucleic acids, primary and intermediary metabolites, carbohydrate polymers, etc. The compounds may 10 be produced by the plant, extracted upon harvest and/or processing, and used for any presently recognized useful purpose such as pharmaceuticals, fragrances, and industrial enzymes to name a few.

Further possibilities to exemplify the range of grain traits or properties potentially encoded by introduced genes in transgenic plants include grain with less breakage susceptibility 15 for export purposes or larger grit size when processed by dry milling through introduction of genes that enhance γ -zein synthesis, popcorn with improved popping quality and expansion volume through genes that increase pericarp thickness, corn with whiter grain for food uses though introduction of genes that effectively block expression of enzymes involved in pigment production pathways, and improved quality of alcoholic beverages or sweet corn through 20 introduction of genes which affect flavor such as the shrunken gene (encoding sucrose synthase) for sweet corn.

7. Plant Agronomic Characteristics

Two of the factors determining where corn can be grown are the average daily temperature during the growing season and the length of time between frosts. Within the areas 25 where it is possible to grow corn, there are varying limitations on the maximal time it is allowed to grow to maturity and be harvested. The corn to be grown in a particular area is selected for its ability to mature and dry down to harvestable moisture content within the required period of time with maximum possible yield. Therefore, corn of varying maturities is developed for different growing locations. Apart from the need to dry down sufficiently to 30 permit harvest, it is desirable to have maximal drying take place in the field to minimize the amount of energy required for additional drying post-harvest. Also, the more readily the grain can dry down, the more time there is available for growth and kernel fill. It is considered that

genes that influence maturity and/or dry down can be identified and introduced into corn lines using transformation techniques to create new corn varieties adapted to different growing locations or the same growing location, but having improved yield to moisture ratio at harvest. Expression of genes that are involved in regulation of plant development may be especially 5 useful, e.g., the liguleless and rough sheath genes that have been identified in corn.

It is contemplated that genes may be introduced into monocots that would improve standability and other plant growth characteristics. Expression of novel genes which confer stronger stalks, improved root systems, or prevent or reduce ear dropage would be of great value to the farmer. It is proposed that introduction and expression of genes that increase the 10 total amount of photoassimilate available by, for example, increasing light distribution and/or interception would be advantageous. In addition, the expression of genes that increase the efficiency of photosynthesis and/or the leaf canopy would further increase gains in productivity. It is contemplated that expression of a phytochrome gene in corn may be advantageous. Expression of such a gene may reduce apical dominance, confer semidwarfism on a plant, and 15 increase shade tolerance (U.S. Pat. No. 5,268,526). Such approaches would allow for increased plant populations in the field.

Delay of late season vegetative senescence would increase the flow of assimilate into the grain and thus increase yield. It is proposed that overexpression of genes within corn that are associated with "stay green" or the expression of any gene that delays senescence would be 20 advantageous. For example, a nonyellowing mutant has been identified in *Festuca pratensis* (Davies *et al.*, 1990). Expression of this gene as well as others may prevent premature breakdown of chlorophyll and thus maintain canopy function.

8. Nutrient Utilization

The ability to utilize available nutrients may be a limiting factor in growth of 25 monocotyledonous plants such as maize. It is proposed that it would be possible to alter nutrient uptake, tolerate pH extremes, mobilization through the plant, storage pools, and availability for metabolic activities by the introduction of novel genes. These modifications would allow a plant such as maize to more efficiently utilize available nutrients. It is contemplated that an increase in the activity of, for example, an enzyme that is normally present 30 in the plant and involved in nutrient utilization would increase the availability of a nutrient. An example of such an enzyme would be phytase. It is further contemplated that enhanced nitrogen utilization by the corn plant is desirable. Expression of a glutamate dehydrogenase

gene in maize, *e.g.*, *E. coli gdhA* genes, may lead to increased fixation of nitrogen in organic compounds. Furthermore, expression of *gdhA* in corn may lead to enhanced resistance to the herbicide glufosinate by incorporation of excess ammonia into glutamate, thereby detoxifying the ammonia. It also is contemplated that expression of a novel gene may make a nutrient source available that was previously not accessible, *e.g.*, an enzyme that releases a component of nutrient value from a more complex molecule, perhaps a macromolecule.

9. Male Sterility

Male sterility is useful in the production of hybrid seed. It is proposed that male sterility may be produced through expression of novel genes. For example, it has been shown that expression of genes that encode proteins that interfere with development of the male inflorescence and/or gametophyte result in male sterility. Chimeric ribonuclease genes that express in the anthers of transgenic tobacco and oilseed rape have been demonstrated to lead to male sterility (Mariani *et al.*, 1990).

A number of mutations were discovered in maize that confer cytoplasmic male sterility. One mutation in particular, referred to as T cytoplasm, also correlates with sensitivity to Southern corn leaf blight. A DNA sequence, designated TURF-13 (Levings, 1990), was identified that correlates with T cytoplasm. It is proposed that it would be possible through the introduction of TURF-13 via transformation, to separate male sterility from disease sensitivity. As it is necessary to be able to restore male fertility for breeding purposes and for grain production, it is proposed that genes encoding restoration of male fertility may also be introduced.

10. Negative Selectable Markers

Introduction of genes encoding traits that can be selected against may be useful for eliminating undesirable linked genes. It is contemplated that when two or more genes are introduced together by cotransformation that the genes will be linked together on the host chromosome. For example, a gene encoding a *Bt* gene that confers insect resistance on the plant may be introduced into a plant together with a *bar* gene that is useful as a selectable marker and confers resistance to the herbicide Liberty® on the plant. However, it may not be desirable to have an insect resistant plant that also is resistant to the herbicide Liberty®. It is proposed that one could also introduce an antisense *bar* gene that is expressed in those tissues where one does not want expression of the *bar* gene, *e.g.*, in whole plant parts. Hence, although

the *bar* gene is expressed and is useful as a selectable marker, it is not useful to confer herbicide resistance on the whole plant. The *bar* antisense gene is a negative selectable marker.

It also is contemplated that negative selection is necessary in order to screen a population of transformants for rare homologous recombinants generated through gene targeting. For example, a homologous recombinant may be identified through the inactivation of a gene that was previously expressed in that cell. The antisense gene to neomycin phosphotransferase II (NPT II) has been investigated as a negative selectable marker in tobacco (*Nicotiana tabacum*) and *Arabidopsis thaliana* (Xiang. and Guerra, 1993). In this example, both sense and antisense NPT II genes are introduced into a plant through transformation and the resultant plants are sensitive to the antibiotic kanamycin. An introduced gene that integrates into the host cell chromosome at the site of the antisense NPT II gene, and inactivates the antisense gene, will make the plant resistant to kanamycin and other aminoglycoside antibiotics. Therefore, rare, site-specific recombinants may be identified by screening for antibiotic resistance. Similarly, any gene, native to the plant or introduced through transformation, that when inactivated confers resistance to a compound, may be useful as a negative selectable marker.

It is contemplated that negative selectable markers may also be useful in other ways. One application is to construct transgenic lines in which one could select for transposition to unlinked sites. In the process of tagging it is most common for the transposable element to move to a genetically linked site on the same chromosome. A selectable marker for recovery of rare plants in which transposition has occurred to an unlinked locus would be useful. For example, the enzyme cytosine deaminase may be useful for this purpose (Stougaard, 1993). In the presence of this enzyme the compound 5-fluorocytosine is converted to 5-fluorouracil which is toxic to plant and animal cells. If a transposable element is linked to the gene for the enzyme cytosine deaminase, one may select for transposition to unlinked sites by selecting for transposition events in which the resultant plant is now resistant to 5-fluorocytosine. The parental plants and plants containing transpositions to linked sites will remain sensitive to 5-fluorocytosine. Resistance to 5-fluorocytosine is due to loss of the cytosine deaminase gene through genetic segregation of the transposable element and the cytosine deaminase gene. Other genes that encode proteins that render the plant sensitive to a certain compound will also be useful in this context. For example, T-DNA gene 2 from *Agrobacterium tumefaciens* encodes a protein that catalyzes the conversion of α -naphthalene acetamide (NAM) to α -

naphthalene acetic acid (NAA) renders plant cells sensitive to high concentrations of NAM (Depicker *et al.*, 1988).

It also is contemplated that negative selectable markers may be useful in the construction of transposon tagging lines. For example, by marking an autonomous transposable element such as Ac, Master Mu, or En/Spn with a negative selectable marker, one could select for transformants in which the autonomous element is not stably integrated into the genome. It is proposed that this would be desirable, for example, when transient expression of the autonomous element is desired to activate in trans the transposition of a defective transposable element, such as Ds, but stable integration of the autonomous element is not desired. The presence of the autonomous element may not be desired in order to stabilize the defective element, *i.e.*, prevent it from further transposing. However, it is proposed that if stable integration of an autonomous transposable element is desired in a plant the presence of a negative selectable marker may make it possible to eliminate the autonomous element during the breeding process.

15 (vi) *Non-Protein-Expressing Sequences*

1. RNA-Expressing

DNA may be introduced into corn and other monocots for the purpose of expressing RNA transcripts that function to affect plant phenotype yet are not translated into protein. Two examples are antisense RNA and RNA with ribozyme activity. Both may serve possible functions in reducing or eliminating expression of native or introduced plant genes.

Genes may be constructed or isolated, which when transcribed, produce antisense RNA that is complementary to all or part(s) of a targeted messenger RNA(s). The antisense RNA reduces production of the polypeptide product of the messenger RNA. The polypeptide product may be any protein encoded by the plant genome. The aforementioned genes will be referred to as antisense genes. An antisense gene may thus be introduced into a plant by transformation methods to produce a novel transgenic plant with reduced expression of a selected protein of interest. For example, the protein may be an enzyme that catalyzes a reaction in the plant. Reduction of the enzyme activity may reduce or eliminate products of the reaction which include any enzymatically synthesized compound in the plant such as fatty acids, amino acids, carbohydrates, nucleic acids and the like. Alternatively, the protein may be a storage protein, such as a zein, or a structural protein, the decreased expression of which may lead to changes in seed amino acid composition or plant morphological changes respectively. The possibilities

cited above are provided only by way of example and do not represent the full range of applications.

Genes also may be constructed or isolated, which when transcribed produce RNA enzymes, or ribozymes, which can act as endoribonucleases and catalyze the cleavage of RNA molecules with selected sequences. The cleavage of selected messenger RNAs can result in the reduced production of their encoded polypeptide products. These genes may be used to prepare novel transgenic plants which possess them. The transgenic plants may possess reduced levels of polypeptides including, but not limited to, the polypeptides cited above that may be affected by antisense RNA.

It also is possible that genes may be introduced to produce novel transgenic plants which have reduced expression of a native gene product by a mechanism of co-suppression. It has been demonstrated in tobacco, tomato, and petunia (Goring *et al.*, 1991; Smith *et al.*, 1990; Napoli *et al.*, 1990; van der Krol *et al.*, 1990) that expression of the sense transcript of a native gene will reduce or eliminate expression of the native gene in a manner similar to that observed for antisense genes. The introduced gene may encode all or part of the targeted native protein but its translation may not be required for reduction of levels of that native protein.

2. Non-RNA-Expressing

DNA elements including those of transposable elements such as Ds, Ac, or Mu, may be inserted into a gene to cause mutations. These DNA elements may be inserted in order to inactivate (or activate) a gene and thereby "tag" a particular trait. In this instance the transposable element does not cause instability of the tagged mutation, because the utility of the element does not depend on its ability to move in the genome. Once a desired trait is tagged, the introduced DNA sequence may be used to clone the corresponding gene, *e.g.*, using the introduced DNA sequence as a PCR primer together with PCR gene cloning techniques (Shapiro, 1983; Dellaporta *et al.*, 1988). Once identified, the entire gene(s) for the particular trait, including control or regulatory regions where desired, may be isolated, cloned and manipulated as desired. The utility of DNA elements introduced into an organism for purposes of gene tagging is independent of the DNA sequence and does not depend on any biological activity of the DNA sequence, *i.e.*, transcription into RNA or translation into protein. The sole function of the DNA element is to disrupt the DNA sequence of a gene.

It is contemplated that unexpressed DNA sequences, including novel synthetic sequences, could be introduced into cells as proprietary "labels" of those cells and plants and

seeds thereof. It would not be necessary for a label DNA element to disrupt the function of a gene endogenous to the host organism, as the sole function of this DNA would be to identify the origin of the organism. For example, one could introduce a unique DNA sequence into a plant and this DNA element would identify all cells, plants, and progeny of these cells as having arisen from that labeled source. It is proposed that inclusion of label DNAs would enable one to distinguish proprietary germplasm or germplasm derived from such, from unlabelled germplasm.

Another possible element which may be introduced is a matrix attachment region element (MAR), such as the chicken lysozyme A element (Stief, 1989), which can be positioned around an expressible gene of interest to effect an increase in overall expression of the gene and diminish position dependent effects upon incorporation into the plant genome (Stief *et al.*, 1989; Phi-Van *et al.*, 1990).

VII. Production and Characterization of Transgenic Plants

After effecting delivery of exogenous DNA to recipient cells, the next steps generally concern identifying the transformed cells for further culturing and plant regeneration. As disclosed herein, in order to improve the ability to identify transformants, one may desire to employ a selectable or screenable marker gene as, or in addition to, the expressible gene of interest. In this case, one would then generally assay the potentially transformed cell population by exposing the cells to a selective agent or agents, or one would screen the cells for the desired marker gene trait.

(i) *Selection*

An exemplary embodiment of methods for identifying transformed cells involves exposing the bombarded cultures to a selective agent, such as a metabolic inhibitor, an antibiotic, herbicide or the like. Cells which have been transformed and have stably integrated a marker gene conferring resistance to the selective agent used, will grow and divide in culture. Sensitive cells will not be amenable to further culturing.

To use the *bar*-bialaphos or the EPSPS-glyphosate selective system, bombarded tissue is cultured for 0 - 28 days on nonselective medium and subsequently transferred to medium containing from 1-3 mg/l bialaphos or 1-3 mM glyphosate as appropriate. While ranges of 1-3 mg/l bialaphos or 1-3 mM glyphosate will typically be preferred, it is proposed that ranges of 0.1-50 mg/l bialaphos or 0.1-50 mM glyphosate may find utility in the practice of the invention.

Tissue can be placed on any porous, inert, solid or semi-solid support for bombardment, including but not limited to filters and solid culture medium. Bialaphos and glyphosate are provided as examples of agents suitable for selection of transformants, but the techniques of this invention are not limited to them as any suitable agents may be used.

5 An example of a screenable marker trait is the red pigment produced under the control of the R-locus in maize. This pigment may be detected by culturing cells on a solid support containing nutrient media capable of supporting growth at this stage and selecting cells from colonies (visible aggregates of cells) that are pigmented. These cells may be cultured further, either in suspension or on solid media. In a similar fashion, the introduction of the C1 and B genes will result in pigmented cells and/or tissues.

10 The enzyme luciferase may be used as a screenable marker in the context of the present invention. In the presence of the substrate luciferin, cells expressing luciferase emit light which can be detected on photographic or x-ray film, in a luminometer (or liquid scintillation counter), by devices that enhance night vision, or by a highly light sensitive video camera, such as a 15 photon counting camera. All of these assays are nondestructive and transformed cells may be cultured further following identification. The photon counting camera is especially valuable as it allows one to identify specific cells or groups of cells which are expressing luciferase and manipulate those in real time. Another particularly useful screenable marker which may be used with the current invention is the gene coding for green fluorescent protein (GFP). GFP is 20 deemed particularly useful in that it may be detected without destruction of the assayed tissue, and because real-time observations may be made (Sheen *et al.*, 1995).

25 It further is contemplated that combinations of screenable and selectable markers will be useful for identification of transformed cells. In some cell or tissue types a selection agent, such as bialaphos or glyphosate, may either not provide enough killing activity to clearly recognize transformed cells or may cause substantial nonselective inhibition of transformants and nontransformants alike, thus causing the selection technique to not be effective. It is proposed that selection with a growth inhibiting compound, such as bialaphos or glyphosate at concentrations below those that cause 100% inhibition followed by screening of growing tissue 30 for expression of a screenable marker gene such as luciferase would allow one to recover transformants from cell or tissue types that are not amenable to selection alone. It is proposed that combinations of selection and screening may enable one to identify transformants in a wider variety of cell and tissue types.

When higher frequencies of transformation are achieved, especially with the use of immature embryos as a target tissue, it is possible that chimeric transformed callus will be produced, *i.e.*, transformants comprising cells that arose from more than one initial transformed cell. It is believed chimeric transformed callus may be avoided by subculturing smaller pieces 5 of the transformed callus, thereby subcloning transformants.

(ii) *Regeneration and Seed Production*

Cells that survive the exposure to the selective agent, or cells that have been scored positive in a screening assay, may be cultured in media that supports regeneration of plants. In an exemplary embodiment, MS and N6 media may be modified (see Table 1) by including further substances such as growth regulators. A preferred growth regulator for such purposes is dicamba or 2,4-D. However, other growth regulators may be employed, including NAA, NAA + 2,4-D or perhaps even picloram. Media improvement in these and similar ways have been found to facilitate the growth of cells at specific developmental stages. Tissue may be maintained on a basic media with growth regulators until sufficient tissue is available to begin 10 plant regeneration efforts, or following repeated rounds of manual selection until the morphology of the tissue is suitable for regeneration, at least two weeks, then transferred to media conducive to maturation of embryoids. Cultures are transferred about every two weeks 15 on this medium, although the time between subcultures may vary depending on the rate of growth of the cells. For example, cultures may be transferred at 3 week intervals. Shoot development will signal the time to transfer to medium lacking growth regulators. 20

The transformed cells, identified by selection or screening and cultured in an appropriate medium that supports regeneration, then will be allowed to mature into plants. Developing plantlets are transferred to soilless plant growth mix, and hardened, *e.g.*, in an environmentally controlled chamber at about 85% relative humidity, 600 ppm CO₂, and 25-250 25 microeinsteins m⁻² s⁻¹ of light. Plants preferably are matured either in a growth chamber or greenhouse. Plants are regenerated from about 6 weeks to 10 months after a transformant is identified, depending on the initial tissue. During regeneration, cells are grown on solid media in tissue culture vessels. Illustrative embodiments of such vessels are petri dishes, Plant Cons and Phytatrays. Regenerating plants preferably are grown at about 19°C to 28°C. After the 30 regenerating plants have reached the stage of shoot and root development, they may be transferred to a greenhouse for further growth and testing.

It should be noted, however, that kernels on transformed plants may occasionally require embryo rescue due to cessation of kernel development and premature senescence of plants. To rescue developing embryos, they are excised from surface-disinfected kernels 10-20 days post-pollination and cultured. An embodiment of media used for culture at this stage comprises MS salts, 2% sucrose, and 5.5 g/l agarose. In embryo rescue, large embryos (defined as greater than 3 mm in length) are germinated directly on an appropriate media. Smaller embryos may be cultured for one week on media containing the above ingredients along with 10^{-5} M abscisic acid and then transferred to growth regulator-free medium for germination.

Progeny may be recovered from the transformed plants and tested for expression of the exogenous expressible gene by localized application of an appropriate substrate to plant parts such as leaves. In the case of *bar* transformed plants, it was found that transformed parental plants (R_O) and their progeny (R_1) exhibited no herbicide-related necrosis after localized application of the herbicide glufosinate (LIBERTY®) to leaves, if there was functional PAT activity in the plants as assessed by an *in vitro* enzymatic assay. All PAT positive progeny tested contained *bar*, confirming that the presence of the enzyme and the resistance to bialaphos were associated with the transmission through the germline of the marker gene.

VIII. Genetic Analysis of Transgenic Plants

In particular embodiments of the invention, methods may be used for detecting the presence or expression of transgenes. The method of assaying expression may comprise determining the level of protein expressed by the transgene or by determining specific alterations in the expressed product. Such assays may in some cases be faster, more accurate or less expensive than conventional screening assays.

The biological sample may potentially be any type of plant tissue. Nucleic acid may be isolated from cells contained in the biological sample, according to standard methodologies (Sambrook *et al.*, 1989). The nucleic acid may be genomic DNA or fractionated or whole cell RNA. Where RNA is used, it may be desired to convert the RNA to a complementary DNA. In one embodiment, the RNA is whole cell RNA; in another, it is poly-A RNA. Normally, the nucleic acid is amplified.

Depending on the format, the specific nucleic acid of interest is identified in the sample directly using amplification or with a second, known nucleic acid following amplification. Next, the identified product is detected. In certain applications, the detection may be performed

by visual means (*e.g.*, ethidium bromide staining of a gel). Alternatively, the detection may involve indirect identification of the product via chemiluminescence, radioactive scintigraphy of radiolabel or fluorescent label or even via a system using electrical or thermal impulse signals (Affymax Technology; Bellus, 1994).

5 Following detection, one may compare the results seen in a given plant with a statistically significant reference group of non-transformed control plants. Typically, the non-transformed control plants will be of a genetic background similar to the transformed plants. In this way, it is possible to detect differences in the amount or kind of protein detected in various transformed plants.

10 A variety of different assays are contemplated in the screening of transgenic plants created using the methods of the current invention. These techniques can be used to detect for both the presence of particular genes as well as rearrangements that may have occurred in the gene construct. The techniques include but are not limited to, fluorescent *in situ* hybridization (FISH), direct DNA sequencing, PFGE analysis, Southern or Northern blotting, single-stranded 15 conformation analysis (SSCA), RNase protection assay, allele-specific oligonucleotide (ASO), dot blot analysis, denaturing gradient gel electrophoresis, RFLP and PCR-SSCP.

(i) *Primers and Probes*

20 The term primer, as defined herein, is meant to encompass any nucleic acid that is capable of priming the synthesis of a nascent nucleic acid in a template-dependent process. Typically, primers are oligonucleotides from ten to twenty base pairs in length, but longer sequences can be employed. Primers may be provided in double-stranded or single-stranded form, although the single-stranded form is preferred. Probes are defined differently, although they may act as primers. Probes, while perhaps capable of priming, are designed to bind to the target DNA or RNA and need not be used in an amplification process.

25 In preferred embodiments, the probes or primers are labeled with radioactive species (^{32}P , ^{14}C , ^{35}S , ^3H , or other label), with a fluorophore (rhodamine, fluorescein), an antigen (biotin, streptavidin, digoxigenin), or a chemilluminiscent (luciferase).

(ii) *Template Dependent Amplification Methods*

30 A number of template dependent processes are available to amplify the marker sequences present in a given template sample. One of the best known amplification methods is the polymerase chain reaction (referred to as PCRTM) which is described in detail in U.S. Pat.

Nos. 4,683,195, 4,683,202 and 4,800,159, each of which is incorporated herein by reference in its entirety.

Briefly, in PCR, two primer sequences are prepared that are complementary to regions on opposite complementary strands of the marker sequence. An excess of deoxynucleoside triphosphates are added to a reaction mixture along with a DNA polymerase, e.g., *Taq* polymerase. If the marker sequence is present in a sample, the primers will bind to the marker and the polymerase will cause the primers to be extended along the marker sequence by adding on nucleotides. By raising and lowering the temperature of the reaction mixture, the extended primers will dissociate from the marker to form reaction products, excess primers will bind to the marker and to the reaction products and the process is repeated.

A reverse transcriptase PCR amplification procedure may be performed in order to quantify the amount of mRNA amplified. Methods of reverse transcribing RNA into cDNA are well known and described in Sambrook *et al.*, 1989. Alternative methods for reverse transcription utilize thermostable, RNA-dependent DNA polymerases and are described in WO 15 90/07641, filed December 21, 1990. Polymerase chain reaction methodologies are well known in the art.

Another method for amplification is the ligase chain reaction ("LCR"), disclosed in EPO No. 320 308, incorporated herein by reference in its entirety. In LCR, two complementary probe pairs are prepared, and in the presence of the target sequence, each pair will bind to opposite complementary strands of the target such that they abut. In the presence of a ligase, the two probe pairs will link to form a single unit. By temperature cycling, as in PCR, bound ligated units dissociate from the target and then serve as "target sequences" for ligation of excess probe pairs. U.S. Pat. No. 4,883,750 describes a method similar to LCR for binding probe pairs to a target sequence.

Qbeta Replicase, described in PCT Application No. PCT/US87/00880, may also be used as still another amplification method in the present invention. In this method, a replicative sequence of RNA that has a region complementary to that of a target is added to a sample in the presence of an RNA polymerase. The polymerase will copy the replicative sequence that can then be detected.

An isothermal amplification method, in which restriction endonucleases and ligases are used to achieve the amplification of target molecules that contain nucleotide 5'-[alpha-thio]-

triphosphates in one strand of a restriction site may also be useful in the amplification of nucleic acids in the present invention, Walker *et al.*, (1992).

Strand Displacement Amplification (SDA) is another method of carrying out isothermal amplification of nucleic acids which involves multiple rounds of strand displacement and synthesis, *i.e.*, nick translation. A similar method, called Repair Chain Reaction (RCR), involves annealing several probes throughout a region targeted for amplification, followed by a repair reaction in which only two of the four bases are present. The other two bases can be added as biotinylated derivatives for easy detection. A similar approach is used in SDA. Target specific sequences can also be detected using a cyclic probe reaction (CPR). In CPR, a probe having 3' and 5' sequences of non-specific DNA and a middle sequence of specific RNA is hybridized to DNA that is present in a sample. Upon hybridization, the reaction is treated with RNase H, and the products of the probe identified as distinctive products that are released after digestion. The original template is annealed to another cycling probe and the reaction is repeated.

Still another amplification methods described in GB Application No. 2 202 328, and in PCT Application No. PCT/US89/01025, each of which is incorporated herein by reference in its entirety, may be used in accordance with the present invention. In the former application, "modified" primers are used in a PCR-like, template- and enzyme-dependent synthesis. The primers may be modified by labeling with a capture moiety (*e.g.*, biotin) and/or a detector moiety (*e.g.*, enzyme). In the latter application, an excess of labeled probes are added to a sample. In the presence of the target sequence, the probe binds and is cleaved catalytically. After cleavage, the target sequence is released intact to be bound by excess probe. Cleavage of the labeled probe signals the presence of the target sequence.

Other nucleic acid amplification procedures include transcription-based amplification systems (TAS), including nucleic acid sequence based amplification (NASBA) and 3SR (Kwoh *et al.*, 1989; Gingeras *et al.*, PCT Application WO 88/10315, incorporated herein by reference in their entirety). In NASBA, the nucleic acids can be prepared for amplification by standard phenol/chloroform extraction, heat denaturation of a clinical sample, treatment with lysis buffer and minispin columns for isolation of DNA and RNA or guanidinium chloride extraction of RNA. These amplification techniques involve annealing a primer which has target specific sequences. Following polymerization, DNA/RNA hybrids are digested with RNase H while double stranded DNA molecules are heat denatured again. In either case, the single stranded

DNA is made fully double stranded by addition of second target specific primer, followed by polymerization. The double-stranded DNA molecules are then multiply transcribed by an RNA polymerase such as T7 or SP6. In an isothermal cyclic reaction, the RNA's are reverse transcribed into single stranded DNA, which is then converted to double stranded DNA, and then transcribed once again with an RNA polymerase such as T7 or SP6. The resulting products, whether truncated or complete, indicate target specific sequences.

Davey *et al.*, EPO No. 329 822 (incorporated herein by reference in its entirety) disclose a nucleic acid amplification process involving cyclically synthesizing single-stranded RNA ("ssRNA"), ssDNA, and double-stranded DNA (dsDNA), which may be used in accordance with the present invention. The ssRNA is a template for a first primer oligonucleotide, which is elongated by reverse transcriptase (RNA-dependent DNA polymerase). The RNA is then removed from the resulting DNA:RNA duplex by the action of ribonuclease H (RNase H, an RNase specific for RNA in duplex with either DNA or RNA). The resultant ssDNA is a template for a second primer, which also includes the sequences of an RNA polymerase promoter (exemplified by T7 RNA polymerase) 5' to its homology to the template. This primer is then extended by DNA polymerase (exemplified by the large "Klenow" fragment of *E. coli* DNA polymerase I), resulting in a double-stranded DNA ("dsDNA") molecule, having a sequence identical to that of the original RNA between the primers and having additionally, at one end, a promoter sequence. This promoter sequence can be used by the appropriate RNA polymerase to make many RNA copies of the DNA. These copies can then re-enter the cycle leading to very swift amplification. With proper choice of enzymes, this amplification can be done isothermally without addition of enzymes at each cycle. Because of the cyclical nature of this process, the starting sequence can be chosen to be in the form of either DNA or RNA.

Miller *et al.*, PCT Application WO 89/06700 (incorporated herein by reference in its entirety) disclose a nucleic acid sequence amplification scheme based on the hybridization of a promoter/primer sequence to a target single-stranded DNA ("ssDNA") followed by transcription of many RNA copies of the sequence. This scheme is not cyclic, *i.e.*, new templates are not produced from the resultant RNA transcripts. Other amplification methods include "RACE" and "one-sided PCR" (Frohman, M.A., In: *PCR PROTOCOLS: A GUIDE TO METHODS AND APPLICATIONS*, Academic Press, N.Y., 1990; Ohara *et al.*, 1989; each herein incorporated by reference in their entirety).

Methods based on ligation of two (or more) oligonucleotides in the presence of nucleic acid having the sequence of the resulting "di-oligonucleotide", thereby amplifying the di-oligonucleotide, may also be used in the amplification step of the present invention. Wu *et al.*, (1989), incorporated herein by reference in its entirety.

5 (iii) *Southern/Northern Blotting*

Blotting techniques are well known to those of skill in the art. Southern blotting involves the use of DNA as a target, whereas Northern blotting involves the use of RNA as a target. Each provide different types of information, although cDNA blotting is analogous, in many aspects, to blotting or RNA species.

10 Briefly, a probe is used to target a DNA or RNA species that has been immobilized on a suitable matrix, often a filter of nitrocellulose. The different species should be spatially separated to facilitate analysis. This often is accomplished by gel electrophoresis of nucleic acid species followed by "blotting" on to the filter.

15 Subsequently, the blotted target is incubated with a probe (usually labeled) under conditions that promote denaturation and rehybridization. Because the probe is designed to base pair with the target, the probe will bind a portion of the target sequence under renaturing conditions. Unbound probe is then removed, and detection is accomplished as described above.

20 (iv) *Separation Methods*

It normally is desirable, at one stage or another, to separate the amplification product from the template and the excess primer for the purpose of determining whether specific amplification has occurred. In one embodiment, amplification products are separated by agarose, agarose-acrylamide or polyacrylamide gel electrophoresis using standard methods. See Sambrook *et al.*, 1989.

25 Alternatively, chromatographic techniques may be employed to effect separation. There are many kinds of chromatography which may be used in the present invention: adsorption, partition, ion-exchange and molecular sieve, and many specialized techniques for using them including column, paper, thin-layer and gas chromatography (Freifelder, 1982).

30 (v) *Detection Methods*

Products may be visualized in order to confirm amplification of the marker sequences. One typical visualization method involves staining of a gel with ethidium bromide and visualization under UV light. Alternatively, if the amplification products are integrally labeled

with radio- or fluorometrically-labeled nucleotides, the amplification products can then be exposed to x-ray film or visualized under the appropriate stimulating spectra, following separation.

In one embodiment, visualization is achieved indirectly. Following separation of 5 amplification products, a labeled nucleic acid probe is brought into contact with the amplified marker sequence. The probe preferably is conjugated to a chromophore but may be radiolabeled. In another embodiment, the probe is conjugated to a binding partner, such as an antibody or biotin, and the other member of the binding pair carries a detectable moiety.

In one embodiment, detection is by a labeled probe. The techniques involved are well 10 known to those of skill in the art and can be found in many standard books on molecular protocols (see Sambrook *et al.*, 1989). For example, chromophore or radiolabel probes or primers identify the target during or following amplification.

One example of the foregoing is described in U.S. Pat. No. 5,279,721, incorporated by 15 reference herein, which discloses an apparatus and method for the automated electrophoresis and transfer of nucleic acids. The apparatus permits electrophoresis and blotting without external manipulation of the gel and is ideally suited to carrying out methods according to the present invention.

In addition, the amplification products described above may be subjected to sequence 20 analysis to identify specific kinds of variations using standard sequence analysis techniques. Within certain methods, exhaustive analysis of genes is carried out by sequence analysis using primer sets designed for optimal sequencing (Pignon *et al.*, 1994). The present invention provides methods by which any or all of these types of analyses may be used.

(vi) *Design and Theoretical Considerations for Relative Quantitative RT-PCR*

25 Reverse transcription (RT) of RNA to cDNA followed by relative quantitative PCR (RT-PCR) can be used to determine the relative concentrations of specific mRNA species isolated from plants. By determining that the concentration of a specific mRNA species varies, it is shown that the gene encoding the specific mRNA species is differentially expressed.

In PCR, the number of molecules of the amplified target DNA increase by a factor 30 approaching two with every cycle of the reaction until some reagent becomes limiting. Thereafter, the rate of amplification becomes increasingly diminished until there is no increase in the amplified target between cycles. If a graph is plotted in which the cycle number is on the

X axis and the log of the concentration of the amplified target DNA is on the Y axis, a curved line of characteristic shape is formed by connecting the plotted points. Beginning with the first cycle, the slope of the line is positive and constant. This is said to be the linear portion of the curve. After a reagent becomes limiting, the slope of the line begins to decrease and eventually becomes zero. At this point the concentration of the amplified target DNA becomes asymptotic to some fixed value. This is said to be the plateau portion of the curve.

The concentration of the target DNA in the linear portion of the PCR amplification is directly proportional to the starting concentration of the target before the reaction began. By determining the concentration of the amplified products of the target DNA in PCR reactions that have completed the same number of cycles and are in their linear ranges, it is possible to determine the relative concentrations of the specific target sequence in the original DNA mixture. If the DNA mixtures are cDNAs synthesized from RNAs isolated from different tissues or cells, the relative abundances of the specific mRNA from which the target sequence was derived can be determined for the respective tissues or cells. This direct proportionality between the concentration of the PCR products and the relative mRNA abundances is only true in the linear range of the PCR reaction.

The final concentration of the target DNA in the plateau portion of the curve is determined by the availability of reagents in the reaction mix and is independent of the original concentration of target DNA. Therefore, the first condition that must be met before the relative abundances of a mRNA species can be determined by RT-PCR for a collection of RNA populations is that the concentrations of the amplified PCR products must be sampled when the PCR reactions are in the linear portion of their curves.

The second condition that must be met for an RT-PCR experiment to successfully determine the relative abundances of a particular mRNA species is that relative concentrations of the amplifiable cDNAs must be normalized to some independent standard. The goal of an RT-PCR experiment is to determine the abundance of a particular mRNA species relative to the average abundance of all mRNA species in the sample.

Most protocols for competitive PCR utilize internal PCR standards that are approximately as abundant as the target. These strategies are effective if the products of the PCR amplifications are sampled during their linear phases. If the products are sampled when the reactions are approaching the plateau phase, then the less abundant product becomes relatively over represented. Comparisons of relative abundances made for many different RNA

samples, such as is the case when examining RNA samples for differential expression, become distorted in such a way as to make differences in relative abundances of RNAs appear less than they actually are. This is not a significant problem if the internal standard is much more abundant than the target. If the internal standard is more abundant than the target, then direct 5 linear comparisons can be made between RNA samples.

The above discussion describes theoretical considerations for an RT-PCR assay for plant tissue. The problems inherent in plant tissue samples are that they are of variable quantity (making normalization problematic), and that they are of variable quality (necessitating the co-amplification of a reliable internal control, preferably of larger size than the target). Both of 10 these problems are overcome if the RT-PCR is performed as a relative quantitative RT-PCR with an internal standard in which the internal standard is an amplifiable cDNA fragment that is larger than the target cDNA fragment and in which the abundance of the mRNA encoding the internal standard is roughly 5-100 fold higher than the mRNA encoding the target. This assay measures relative abundance, not absolute abundance of the respective mRNA species.

Other studies may be performed using a more conventional relative quantitative RT-PCR assay with an external standard protocol. These assays sample the PCR products in the linear portion of their amplification curves. The number of PCR cycles that are optimal for sampling must be empirically determined for each target cDNA fragment. In addition, the reverse transcriptase products of each RNA population isolated from the various tissue samples 15 must be carefully normalized for equal concentrations of amplifiable cDNAs. This consideration is very important since the assay measures absolute mRNA abundance. Absolute mRNA abundance can be used as a measure of differential gene expression only in normalized samples. While empirical determination of the linear range of the amplification curve and normalization of cDNA preparations are tedious and time consuming processes, the resulting 20 RT-PCR assays can be superior to those derived from the relative quantitative RT-PCR assay with an internal standard.

One reason for this advantage is that without the internal standard/competitor, all of the reagents can be converted into a single PCR product in the linear range of the amplification 25 curve, thus increasing the sensitivity of the assay. Another reason is that with only one PCR product, display of the product on an electrophoretic gel or another display method becomes less complex, has less background and is easier to interpret.

(vii) *Chip Technologies*

Specifically contemplated by the present inventors are chip-based DNA technologies such as those described by Hacia *et al.* (1996) and Shoemaker *et al.* (1996). Briefly, these techniques involve quantitative methods for analyzing large numbers of genes rapidly and accurately. By tagging genes with oligonucleotides or using fixed probe arrays, one can employ chip technology to segregate target molecules as high density arrays and screen these molecules on the basis of hybridization (see also, Pease *et al.*, 1994; and Fodor *et al.*, 1991).

IX. Examples

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

EXAMPLE 1**Preparation of Microprojectiles**

Microprojectile preparation was as follows: gold particles were prepared by adding 60 mg of 0.6 μ m (BioRad, cat. no. 165-2262) to 1000 μ l absolute ethanol and incubating for at least 3 hours at room temperature followed by storage at -20°C. Twenty to thirty five μ l of the sterile gold particles and more preferably 30 to 35 μ l of gold particles (30 μ l contains 1.8 mg of particles) were centrifuged in a microcentrifuge for up to 1 min. The supernatant was removed and one ml sterile water was added to the tube, followed by centrifugation at 1800-2000 rpm for 2-5 minutes. Microprojectile particles were resuspended in 25-30 μ l of DNA solution containing from 0.0025 μ g to 25 μ g of vector DNA, depending on the treatment, as described in

Examples 3 and 4. Vector DNA used included isolated expression cassettes from plasmids pDPG570 and pDPG756 (FIG. 1 and FIG.2, respectively).

Two hundred twenty microliters sterile water, 250 μ l 2.5 M CaCl₂ and 50 μ l stock spermidine (14 μ l spermidine in 986 μ l water) then were added to the particle containing solution. The solution was then thoroughly mixed and placed on ice, followed by vortexing at 4°C for 10 minutes and centrifugation at 500 rpm for 5 minutes. The supernatant was removed and the pellet resuspended in 600 μ l absolute ethanol. Following centrifugation at 500 rpm for 5 minutes, the pellet was resuspended in 36-38 μ l of absolute ethanol, vortexed for approximately 20 seconds, and sonicated for 20-30 seconds. At this stage the particles may be 5 allowed to sit for 2-5 minutes, after which 5-10 μ l of the supernatant was removed and dispensed on the surface of a flyer disk and the ethanol was allowed to dry completely. Alternatively, particles may be removed directly after resuspension and vortexing 20 to 30 10 seconds in 36 μ l-38 μ l of ethanol, placed on the flyer disk and allowed to dry as done for the settled treatment. The bombardment chamber is then evacuated to approximately 28 in. Hg 15 prior to bombardment. The particles were then used for bombardment by a helium blast of approximately 1100 psi using the DuPont Biolistics PDS1000He particle bombardment device.

EXAMPLE 2

Bombardment of Hi-II Immature Embryos

Immature embryos of the corn genotype Hi-II were used to study the effect of DNA concentration and dephosphorylation of transforming DNA prior to microprojectile bombardment. Immature embryos (1.2 - 3.0 mm in length) were excised from surface-sterilized, greenhouse-grown ears of Hi-II 10-12 days post-pollination. The Hi-II genotype was developed from an A188 x B73 cross (Armstrong *et al.*, 1991). Approximately 30 embryos per 20 petri dish were plated axis side down on a modified N6 medium containing 1 mg/l 2,4-D, 100 mg/l casein hydrolysate, 6 mM L-proline, 0.5 g/l 2-(N-morpholino) ethanesulfonic acid (MES), 0.75 g/l MgCl₂, and 2% sucrose solidified with 2 g/l Gelgro, pH 5.8 (#735 medium). Embryos 25 were cultured in the dark for two to four days at 24°C.

Approximately 3-4 hours prior to bombardment, embryos were transferred to the above 30 culture medium with the sucrose concentration increased from 3% to 12%. When embryos were transferred to the high osmoticum medium they were arranged in concentric circles on the plate, starting 1 cm from the center of the dish, positioned such that their coleorhizal end was

orientated toward the center of the dish. Usually two concentric circles were formed with 25-35 embryos per plate.

The plates containing embryos were placed on the third shelf from the bottom, 5 cm below the stopping screen. The 1100 psi rupture discs were used for bombardment. In separate bombardments, discs were used which contained each of the different transforming DNA compositions described in Examples 3 and 4, *i.e.*, with and without dephosphorylation of transforming DNA and at varying DNA concentrations. Each plate of embryos was bombarded once with the DuPont Biolistics PDS1000He particle gun.

Following bombardment, embryos were allowed to recover on high osmoticum medium (735, 12% sucrose) overnight (16 - 24 hours) and were then transferred to selection medium containing 1 mg/l bialaphos (#739, 735 plus 1 mg/l bialaphos or #750, 735 plus 0.2M mannitol and 1 mg/l bialaphos). Embryos were maintained in the dark at 24° C. After three to four weeks on the initial selection plates about 90% of the embryos had formed Type II callus and were transferred to selective medium containing 3 mg/l bialaphos (#758). Southern analysis was then used to determine transgene copy number.

EXAMPLE 3

Effect of DNA Concentration and Dephosphorylation on Transgene Copy Number

Nine bombardment studies were performed with the actin1P/I-lox5-bar (QD) cassette. The cassette DNA was prepared by restriction digestion of 100 µg of pDPG756 (5.8 kb) (FIG. 2) with HindIII and SnaB1, which liberates the ActinP/I-lox5-bar-Tr7 cassette, and with ScaI to digest the vector backbone into fragments smaller than the cassette. The restriction digest occurred for 2 hours in restriction buffer M (Boehringer Mannheim) at 37°C. A dephosphorylated treatment was then prepared by adding 2.5 U calf intestinal alkaline phosphatase (Boehringer Mannheim, Catalogue No. 713023) to one half of the restriction digest, followed by a 30 minutes incubation at 37°C. DNA from both alkaline phosphatase treated and untreated samples were stored at -20°C overnight until the cassette DNA could be gel purified.

The alkaline phosphatase treated and untreated DNA was then run out in separate lanes on a 1% agarose gel in TAE buffer. A 2.8 kb fragment corresponding to the ActinP/I-lox5-bar-Tr7 cassette was cut out of the gel for each lane. DNA was eluted from the gel slices in dialysis

tubing in TAE buffer for 1 hour at 100V. The concentration of the eluted DNA was determined by spectrophotometry, with an average recovery of 64% of the starting cassette DNA from each sample.

The isolated DNA was used with the procedure of Example 1 to make nine microprojectile preparations. In eight of the nine preparations, the untreated DNA was used and only DNA concentrations were varied, using 10, 20, 100, 100, 100, 800, 1000, and 2000 ng of cassette DNA per each DNA precipitation reaction containing 1.8 mg of 0.6 μ m gold particles. In the ninth study, 1000 ng of the alkaline phosphatase treated DNA was used. Each of the microprojectile compositions were then used individually for bombardment of Hi-II immature embryos. The bombardment procedure followed Example 2.

Sixty-four independent, stable transformation events were recovered 8 weeks after selection on 3 mg/l bialaphos. Genomic DNA was isolated from callus, digested with EcoRI (cuts once in the cassette), electrophoresed on a 0.8% agarose gel (TAE), blotted (MagchargeTM), and screened for copy number by Southern hybridization with a *bar* gene probe at 65° C using the conditions described in Example 8. The bar probe was prepared via PCR amplification with the methods as described in Example 9. The results, given in Table 3, revealed that even at the lowest DNA concentration (10 ng, Study 15), transformants could be selected, and that in general, lowering the DNA concentration increased the likelihood that lower copy events were obtained. However, an even larger effect than DNA concentration was witnessed by use of alkaline phosphatase treated DNA. As shown in Study 26 + AP, using a DNA concentration of 1000 ng, 5 of the 7 transformation events (26.1, 26.2, 26.3, 26.7, and 26.6), were single copy events. In a corresponding study using 1000 ng of non-alkaline phosphatase treated DNA (26 -AP), the one transformation event recovered had 5 copies of the transgene. Even in the 6 studies done using less than 1000 ng of untreated DNA (Studies 15, 16, 17, 19, 22, and 24), only 4 of 52 transformation events obtained were single copy. The results, therefore, demonstrate the efficacy of alkaline phosphatase treatment in increasing the relative yield of low-copy transformation events.

Table 3

The effect of DNA concentration and alkaline phosphatase treatment on copy number of the integration event. DNA concentration is the amount of cassette, in ng, that is precipitated onto 0.6 µm gold particles and used for bombardment of 90 to 120 embryos.

| STUDY# | DNA | TOTAL # | #SINGLE | #2-5 | #6-10 | #>10 |
|---------|---------------|---------|----------------|--------|--------|--------|
| | CONC. (NG) | EVENTS | COPY EVENTS | COPIES | COPIES | COPIES |
| 15 | 10 | 2 | 0 | 1 | 1 | 0 |
| 16 | 100 | 15 | 1 | 5 | 5 | 4 |
| 17 | 800 | 13 | 1 | 4 | 6 | 2 |
| 19 | 100 | 4 | 0 | 3 | 1 | 0 |
| 20 | 2000 | 14 | 1 | 5 | 3 | 5 |
| 22 | 100 | 1 | 0 | 1 | 0 | 0 |
| 24 | 20 | 7 | 2 | 4 | 1 | 0 |
| 26 + AP | 1000 | 7 | 5 | 2 | 0 | 0 |
| 26 - AP | 1000 | 1 | 0 | 1 | 0 | 0 |

5

EXAMPLE 4

DNA Concentration and Transgene Copy Number

A second study was designed to evaluate the effect of DNA concentration on transformation efficiency and on transgene copy number. Serial dilutions of cassette DNA from the Bt-gene encoding plasmid pDPG570 (35S/4XOCS-Sh1 leader/intron1-MZTP-HD73 Bt-pin3: 35S-*bar*-Tr7) (FIG. 1) were used. Concentrations from 25 µg - 2.5 ng were analyzed 10 30 µl 1.8 µg 0.6 µ Gold particles. Microprojectiles were prepared according to Example 1 and bombardments were performed according to Example 2.

Results demonstrated that DNA concentrations as low as 25 ng/preparation did not 15 significantly lower transformation efficiency. Southern Blot analysis was performed on transformants with the procedure described in Example 7 using a 16-20 hour *Nco*I digest (New England Biolabs) at 37°C using the manufacturers suggested concentration of enzyme and buffer. The restricted DNA was then blotted to a filter and analyzed for copy number by probing with ³²P labeled DNA of a 500 base pair fragment from the synthetic Bt gene HD73,

which is disclosed in, for example, WO 95/06128 specifically incorporated herein by reference in its entirety.

Six clones recovered from the 25 ng DNA preparation were analyzed. Half of the transformants had a low (1-3) copy number and half had a moderate (3-6) copy number. No high copy number transformants were recovered from this DNA concentration. In contrast, two of the nine clones analyzed from the 25 µg DNA preparation and three of the ten clones analyzed from the 2.5 µg DNA preparation had a high (>6) or very high (>10) copy number. Results indicate that reducing the amount of DNA typically used for bombardment (25 µg) by 1000 fold promotes recovery of transformants with less complex transgene integration patterns (Table 4).

Table 4
Effect of DNA Concentration on Transgene Copy Number

| | | DNA Concentration | | | | |
|------------------|----------------------|--------------------------|---------------|----------------|--------------|---------------|
| | | 25 µg | 2.5 µg | 0.25 µg | 25 ng | 2.5 ng |
| number of | transformants | 10 | 16 | 12 | 8 | 0 |
| Estimate of | | low | low | low | low | |
| Copy Number | for each | medium | low | medium | low | |
| Transformant | | medium | medium | medium | medium | |
| Tested* (not all | | medium | medium | medium | medium | |
| transformants | were tested) | medium | medium | medium | medium | |
| | | medium | medium | high | | |
| | | very high | high | | | |
| | | very high | very high | | | |
| | | | | extremely high | | |

*Copy Number Estimates:

| | |
|----------------|------------|
| Low | 1-3 copies |
| Medium | 3-6 copies |
| High+ | >6 copies |
| Very High | >10 copies |
| Extremely High | >20 copies |

EXAMPLE 5**Blunting of Overhanging Ends**

For blunting of DNA ends with mung bean nuclease, the enzyme is used in an amount sufficient to digest the DNA, for example, 1 unit of enzyme per 1 µg of DNA. However, the amount may vary from 0.5 unit to 2 units or the like depending on the reaction conditions. The enzyme unit is that as defined by the supplier, e.g., Pharmacia P-L Biochemicals. Examples of some other nucleases which could be used include those described in "Nucleases" Ed. Linn & Roberts, Cold Spring Harbor, N.Y. 1982, which is incorporated herein by reference, of particular interest being an article therein entitled "Single Strand Specific Nucleases" by Shishido and Ando at page 167. Typically, the mung bean nuclease reaction is carried out in a volume of 100 µl with 1 unit of enzyme per 1 µg of genomic DNA at about 50 degree(s) C, in a suitable buffer, for example, 0.2M NaCl, 1 mM ZnSO₄ and 30 mM Na-acetate, pH 4.6. The incubation is carried out for a sufficient time, usually about 30 minutes in the above reaction mixture.

The reaction can then be stopped by diluting the solution about 4-fold with about 0.01M ethylenediamine tetraacetate (EDTA), followed by phenol / chloroform extraction, as is well known in the art. The DNA fragments to be used for transformation are then isolated from this reaction mixture by precipitating the DNA fragments with 2 volumes of absolute ethanol. The precipitate is left overnight at -20 degree(s) C., then centrifuged at about 10,000 rpm (12,000g) for about 30 minutes in a refrigerated centrifuge. The precipitate comprising the isolated DNA is rinsed with 80% ethanol in H₂O, the supernatant poured off, the residue re-centrifuged if necessary and then dried in vacuum. The dried DNA is then resuspended to the desired concentration, for example, in 10 mM tris-HCl, pH 7.5 containing 1 mM NaEDTA, and either stored at -20 degree(s) C or used for transformation.

Blunting of DNA ends with *E. coli* DNA polymerase I is carried out as follows: a 1 mM mixture of the four deoxynucleotide triphosphates is prepared in an Eppendorf tube with 5 µl each of 10 mM dATP, dCTP, dGTP, and dTTP and 30 µl H₂O. In a second Eppendorf tube a mix is made consisting of 8.5 µl H₂O, 5 µl DNA solution (containing 1–5 µg DNA), 1.5 µl 1 M Tris-HCl (pH 7.5), 2.5 µl 0.1 M MgCl₂, 2.5 µl 0.1 M 2-mercaptoethanol, 0.5 µl DNA polymerase I (2.5 units) (one unit is defined as the amount of enzyme required to catalyze the incorporation of 10 nmol of total deoxyribonucleotides into TCA insoluble form in 30 minutes

at 37°C in 67 mM potassium phosphate (pH 7.5), 6.7 mM MgCl₂, 1 mM DTT, 133 μM activated calf thymus DNA and 33 μM dAPT, dCTP, dGTP and radiolabeled dTTP), and the solution is incubated at 12°C for 10 minutes. Then 5 μl of the 1 mM deoxynucleotide triphosphate mixture from the first tube is added to the DNA, and the DNA is incubated for 1 hour at 12°C, followed by heating at 68°C for 10 minutes to inactivate the DNA polymerase. The DNA can then be purified by phenol/chloroform extraction and / or ethanol precipitation.

For blunting of ends with the Klenow fragment of *E. coli* DNA polymerase I, 0.1 to 1 μg of transforming DNA is first combined with 2 μl of 10× fill-in buffer (500 mM Tris-HCl at pH 7.5, 100 mM MgCl₂, 10 mM dithiothreitol (DTT), 500 μg/ml bovine serum albumin), 2 mM (3 μl) each of four deoxyribonucleoside triphosphates (dATP, dCTP, dGTP, and TTP), and 1 unit Klenow fragment. The mixture is then incubated for 30 min at 37° C. The reaction can be stopped by adding by adding 1 μl of 0.5 M EDTA (pH 8.0), followed by 100 μl of TE at pH 8.0 (10 mM Tris-HCl at pH 8.0, 1 mM EDTA). The DNA blunted DNA can be extracted with phenol/chloroform, followed by precipitation in 70% ethanol and resuspension in TE. Alternatively, unincorporated triphosphates may be removed using a spun Sephadex G-50 column.

EXAMPLE 6

General Methods for Microprojectile Bombardment

Many variations in techniques for microprojectile bombardment are well known in the art and therefore deemed useful with the current invention. Exemplary procedures for bombardment are discussed in, for example, U.S. Pat. Appl. No. 08/113,561, filed August 25, 1993. Examples of target tissues which may be used with the current invention include immature embryos, Type I callus, Type II callus, Type III callus, suspension cultures and meristematic tissue (PCT Publication WO 96/04392). Some genotypes which are especially useful for maize transformation are disclosed in, for example, U.S. Pat. Appl. No. 08/113,561, filed August 25, 1993. Preferred genotypes will be those which are readily transformable and which may also be regenerated to yield a fertile transgenic plant.

Any method for acceleration of microprojectiles may potentially be used to transform a plant cell with the current invention. A preferred method will be a gas-driven particle gun such as the DuPont Biolistics PDS1000He particle bombardment device. Exemplary particles for bombardment include those comprised of tungsten, gold, platinum, and the like. Gold particles

are deemed particularly useful in the current invention, with 0.6 μm or 0.7 μm gold particles being preferred and 0.6 μm particles most preferred. The most preferred particles will be DNA coated and have a mean size between 0.6 μm and 1.0 μm .

As disclosed herein, any DNA sequence may potentially be used for transformation. 5 The DNA segments used for transformation will preferably include one or more selectable, secretable or screenable markers. Many examples of such are well known in the art and are specifically disclosed herein. In the case of selectable markers, selection may be in solid or liquid media. The DNA segments used will preferably also include one or more genes which confer, either individually or in combination with other sequences, a desired phenotype on the 10 transformed plant. Exemplary genes for transformation and the corresponding phenotypes these sequences may confer on the transformed plant are disclosed herein.

EXAMPLE 7

Introgression of Transgenes Into Elite Inbreds and Hybrids

Backcrossing can be used to improve an inbred plant. Backcrossing transfers a specific 15 desirable trait from one inbred source to an inbred that lacks that trait. This can be accomplished, for example, by first crossing a superior inbred (A) (recurrent parent) to a donor inbred (non-recurrent parent), which carries the appropriate gene(s) for the trait in question. The progeny of this cross first are selected in the resultant progeny for the desired trait to be 20 transferred from the non-recurrent parent, then the selected progeny are mated back to the superior recurrent parent (A). After five or more backcross generations with selection for the desired trait, the progeny are hemizygous for loci controlling the characteristic being transferred, but are like the superior parent for most or almost all other genes. The last backcross generation would be selfed to give progeny which are pure breeding for the gene(s) 25 being transferred, *i.e.* one or more transformation events.

Therefore, through a series of breeding manipulations, a selected transgene may be moved from one line into an entirely different line without the need for further recombinant manipulation. Transgenes are valuable in that they typically behave genetically as any other gene and can be manipulated by breeding techniques in a manner identical to any other corn 30 gene. Therefore, one may produce inbred plants which are true breeding for one or more transgenes. By crossing different inbred plants, one may produce a large number of different hybrids with different combinations of transgenes. In this way, plants may be produced which

have the desirable agronomic properties frequently associated with hybrids ("hybrid vigor"), as well as the desirable characteristics imparted by one or more transgene(s).

EXAMPLE 8

5

Marker Assisted Selection

Genetic markers may be used to assist in the introgression of one or more transgenes from one genetic background into another. Marker assisted selection offers advantages relative to conventional breeding in that it can be used to avoid errors caused by phenotypic variations. Further, genetic markers may provide data regarding the relative degree of elite germplasm in the individual progeny of a particular cross. For example, when a plant with a desired trait which otherwise has a non-agronomically desirable genetic background is crossed to an elite parent, genetic markers may be used to select progeny which not only possess the trait of interest, but also have a relatively large proportion of the desired germplasm. In this way, the number of generations required to introgress one or more traits into a particular genetic background is minimized.

10

In the process of marker assisted breeding, DNA sequences are used to follow desirable agronomic traits (Tanksley *et al.*, 1989) in the process of plant breeding. Marker assisted breeding may be undertaken as follows. Seed of plants with the desired trait are planted in soil in the greenhouse or in the field. Leaf tissue is harvested from the plant for preparation of DNA at any point in growth at which approximately one gram of leaf tissue can be removed from the plant without compromising the viability of the plant. Genomic DNA is isolated using a procedure modified from Shure *et al.* (1983). Approximately one gram of leaf tissue from a seedling is lyophilized overnight in 15 ml polypropylene tubes. Freeze-dried tissue is ground to a powder in the tube using a glass rod. Powdered tissue is mixed thoroughly with 3 ml extraction buffer (7.0 urea, 0.35 M NaCl, 0.05 M Tris-HCl pH 8.0, 0.01 M EDTA, 1% sarcosine). Tissue/buffer homogenate is extracted with 3 ml phenol/chloroform. The aqueous phase is separated by centrifugation, and precipitated twice using 1/10 volume of 4.4 M ammonium acetate pH 5.2, and an equal volume of isopropanol. The precipitate is washed with 75% ethanol and resuspended in 100-500 µl TE (0.01 M Tris-HCl, 0.001 M EDTA, pH 8.0).

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Genomic DNA is then digested with a 3-fold excess of restriction enzymes, electrophoresed through 0.8% agarose (FMC), and transferred (Southern, 1975) to Nytran (Schleicher and Schuell) using 10X SCP (20 SCP: 2M NaCl, 0.6 M disodium phosphate, 0.02

M disodium EDTA). The filters are prehybridized in 6X SCP, 10% dextran sulfate, 2% sarcosine, and 500 µg/ml denatured salmon sperm DNA and ³²P-labeled probe generated by random priming (Feinberg & Vogelstein, 1983). Hybridized filters are washed in 2X SCP, 1% SDS at 65° for 30 minutes and visualized by autoradiography using Kodak XAR5 film.

5 Genetic polymorphisms which are genetically linked to traits of interest are thereby used to predict the presence or absence of the traits of interest.

Those of skill in the art will recognize that there are many different ways to isolate DNA from plant tissues and that there are many different protocols for Southern hybridization that will produce identical results. Those of skill in the art will recognize that a Southern blot can be stripped of radioactive probe following autoradiography and re-probed with a different probe. In this manner one may identify each of the various transgenes that are present in the plant. Further, one of skill in the art will recognize that any type of genetic marker which is polymorphic at the region(s) of interest may be used for the purpose of identifying the relative presence or absence of a trait, and that such information may be used for marker assisted breeding.

Each lane of a Southern blot represents DNA isolated from one plant. Through the use of multiplicity of gene integration events as probes on the same genomic DNA blot, the integration event composition of each plant may be determined. Correlations may be established between the contributions of particular integration events to the phenotype of the plant. Only those plants that contain a desired combination of integration events may be advanced to maturity and used for pollination. DNA probes corresponding to particular transgene integration events are useful markers during the course of plant breeding to identify and combine particular integration events without having to grow the plants and assay the plants for agronomic performance.

It is expected that one or more restriction enzymes will be used to digest genomic DNA, either singly or in combinations. One of skill in the art will recognize that many different restriction enzymes will be useful and the choice of restriction enzyme will depend on the DNA sequence of the transgene integration event that is used as a probe and the DNA sequences in the genome surrounding the transgene. For a probe, one will want to use DNA or RNA sequences which will hybridize to the DNA used for transformation. One will select a restriction enzyme that produces a DNA fragment following hybridization that is identifiable as the transgene integration event. Thus, particularly useful restriction enzymes will be those

which reveal polymorphisms that are genetically linked to specific transgenes or traits of interest.

EXAMPLE 9

General Methods for Assays

5 DNA analysis of transformed plants is performed as follows. Genomic DNA is isolated using a procedure modified from Shure, *et al.*, 1983. Approximately 1 gm callus or leaf tissue is ground to a fine powder in liquid nitrogen using a mortar and pestle. Powdered tissue is mixed thoroughly with 4 ml extraction buffer (7.0 M urea, 0.35 M NaCl, 0.05 M Tris-HCl pH 8.0, 0.01 M EDTA, 1% sarcosine). Tissue/buffer homogenate is extracted with 4 ml phenol/chloroform. The aqueous phase is separated by centrifugation, passed through Miracloth, and precipitated twice using 1/10 volume of 4.4 M ammonium acetate, pH 5.2 and an equal volume of isopropanol. The precipitate is washed with 70% ethanol and resuspended in 200-500 µl TE (0.01 M Tris-HCl, 0.001 M EDTA, pH 8.0).

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15 The presence of a DNA sequence in a transformed cell may be detected through the use of polymerase chain reaction (PCR). Using this technique specific fragments of DNA can be amplified and detected following agarose gel electrophoresis. For example, two hundred to 1000 ng genomic DNA is added to a reaction mix containing 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl₂, 50 mM KCl, 0.1 mg/ml gelatin, 200µM each dATP, dCTP, dGTP, dTTP, 0.5µM each forward and reverse DNA primers, 20% glycerol, and 2.5 units Taq DNA polymerase. The 20 reaction is run in a thermal cycling machine as follows: 3 minutes at 94 C, 39 repeats of the cycle 1 minute at 94 C, 1 minute at 50 C, 30 seconds at 72 C, followed by 5 minutes at 72 C. Twenty µl of each reaction mix is run on a 3.5% NuSieve gel in TBE buffer (90 mM Tris-borate, 2 mM EDTA) at 50V for two to four hours. Using this procedure, for example, one 25 may detect the presence of the *bar* gene, using the forward primer CATCGAGACAAGCACGGTCAACTTC and the reverse primer AAGTCCCTGGAGGCACAGGGCTTCAAGA.

30 A method to detect the presence of phosphinothricin acetyl transferase (PAT) involves the use of an *in vitro* enzyme reaction followed by thin layer chromatography, as described in U.S. Pat. Appl. No. 08/113,561, filed August 25, 1993 (specifically incorporated herein by reference in its entirety). The procedure is conducted by preparing various protein extracts from homogenates of potentially transformed cells, and from control cells that have neither been transformed nor exposed to bialaphos selection, and then assaying by incubation with PPT

and ^{14}C -Acetyl Coenzyme A followed by thin layer chromatography. The results of this assay provide confirmation of the expression of the *bar* gene which codes for PAT.

For Southern blot analysis genomic DNA is digested with a 3-fold excess of restriction enzymes, electrophoresed through 0.8% agarose (FMC), and transferred (Southern, 1975) to Nytran (Schleicher and Schuell) using 10X SCP (20X SCP: 2 M NaCl, 0.6 M disodium phosphate, 0.02 M disodium EDTA). Filters are prehybridized at 65°C in 6X SCP, 10% dextran sulfate, 2% sarcosine, and 500 $\mu\text{g}/\text{ml}$ heparin (Chomet *et al.*, 1987) for 15 min. Filters then are hybridized overnight at 65°C in 6X SCP containing 100 $\mu\text{g}/\text{ml}$ denatured salmon sperm DNA and ^{32}P -labeled probe. Filters are washed in 2X SCP, 1% SDS at 65°C for 30 min. and visualized by autoradiography using Kodak XAR5 film. For rehybridization, the filters are boiled for 10 min. in distilled H_2O to remove the first probe and then prehybridized as described above.

EXAMPLE 10

Utilization of Transgenic Crops

The ultimate goal in plant transformation is to produce plants which are useful to man. In this respect, transgenic plants created in accordance with the current invention may be used for virtually any purpose deemed of value to the grower or to the consumer. For example, one may wish to harvest seed from transgenic plants. This seed may in turn be used for a wide variety of purposes. The seed may be sold to farmers for planting in the field or may be directly used as food, either for animals or humans. Alternatively, products may be made from the seed itself. Examples of products which may be made from the seed include, oil, starch, animal or human food, pharmaceuticals, and various industrial products. Such products may be made from particular plant parts or from the entire plant. One product made from the entire plant, which is deemed of particular value, is silage for animal feed.

Means for preparing products from plants, such as those that may be made with the current invention, have been well known since the dawn of agriculture and will be obvious to those of skill in the art. Specific methods for crop utilization may be found in, for example, Sprague and Dudley (1988), and Watson and Ramstad (1987).

All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred

embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related 5 may be substituted for the agents described herein while the same or similar results would be achieved. For example, various different methods may be employed for dephosphorylating or blunting transforming DNA, and / or reducing DNA concentration, and still not depart from the spirit, scope and concept of the invention as defined by the appended claims.

REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference in their entirety.

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- U.S. Patent No. 4,535,060
- U.S. Patent No. 4,683,195
- U.S. Patent No. 4,683,202
- 10 U.S. Patent No. 4,800,159
- U.S. Patent No. 4,883,750
- U.S. Patent No. 5,134,074
- U.S. Patent No. 5,183,752
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CLAIMS:

1. A method of preparing a transgenic plant comprising:
 - (a) end-modifying a plurality of nucleic acid segments;
 - (b) contacting recipient plant cells with said nucleic acid segments;
 - 5 (c) regenerating a plant from a plant cell which has been stably transformed with at least a first nucleic acid segment.
2. The method of claim 1, wherein said transgenic plant is fertile.
- 10 3. The method of claim 1, wherein said nucleic acid segments are further defined as DNA segments.
4. The method of claim 3, wherein said end-modifying comprises dephosphorylating.
- 15 5. The method of claim 4, wherein said dephosphorylating comprises treating said DNA segments with a phosphatase.
6. The method of claim 5, wherein said phosphatase is calf alkaline phosphatase.
- 20 7. The method of claim 5, wherein said phosphatase is from *Aspergillus niger*.
8. The method of claim 5, wherein said phosphatase is from *Saccharomyces cerevisiae*.
9. The method of claim 4, wherein the plant is a monocot.
- 25 10. The method of claim 9, wherein said monocot is selected from the group consisting of maize, rice, wheat, barley, oats, rye, millet, sorghum, sugarcane and turfgrass.
11. The method of claim 10, wherein the monocot is maize.

12. The method of claims 9, 10, or 11, wherein said contacting comprises microprojectile bombardment.

13. The method of claim 4, wherein the plant is a dicot.

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14. The method of claim 13, wherein said dicot is selected from the group consisting of cotton, soybean, tomato, potato, citrus, and tobacco.

15. The method of claim 14, wherein the dicot is soybean.

10

16. The method of claim 3, wherein said end-modifying comprises blunting the ends of said DNA fragments.

15

17. The method of claim 16, wherein said blunting comprises treating said DNA segments with a DNA polymerase.

18. The method of claim 17, wherein said DNA polymerase is selected from the group consisting of *E. coli* DNA polymerase I, the Klenow fragment of *E. coli* DNA polymerase I, and T_4 DNA polymerase.

20

19. The method of claim 18, wherein said DNA polymerase is a Klenow fragment *E. coli* DNA polymerase I.

25

20. The method of claim 18, wherein said DNA polymerase is *E. coli* DNA polymerase I.

25

21. The method of claim 20, wherein said DNA polymerase is T₁ DNA polymerase.

22. The method of claim 16, wherein said blunting comprises treating said DNA segments with a nuclease capable of selectively degrading single-stranded DNA

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23. The method of claim 22, wherein said DNA nuclease is selected from the group consisting of S1 nuclease, and mung bean nuclease.

24. The method of claim 23, wherein said nuclease is S1 nuclease.

25. The method of 23, wherein said nuclease is mung bean nuclease.

5

26. The method of claim 16, wherein said blunting comprises treating said DNA segments with a blunt-cutting restriction endonuclease.

27. The method of claim 16, wherein the plant is a monocot.

10

28. The method of claim 27, wherein said monocot is selected from the group consisting of maize, rice, wheat, barley, oats, rye, millet, sorghum, sugarcane and turfgrass.

29. The method of claim 28, wherein the monocot is maize.

15

30. The method of claims 27, 28, or 29, wherein said contacting comprises microprojectile bombardment.

31. The method of claim 16, wherein the plant is a dicot.

20

32. The method of claim 31, wherein said dicot is selected from the group consisting of cotton, soybean, tomato, potato, citrus, and tobacco.

33. The method of claim 32, wherein the dicot is soybean.

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34. The method of claim 4 or 16, wherein said contacting comprises microprojectile bombardment, PEG-mediated transformation, or electroporation.

35. The method of claim 34, wherein said contacting comprises microprojectile bombardment.

36. The method of claim 35, wherein said microprojectile bombardment comprises coating microprojectiles with said DNA segments and contacting said recipient plant cells with said microprojectiles.

5 37. The method of claim 34, wherein said contacting comprises electroporation.

38. The method of claim 34, wherein said contacting comprises PEG-mediated transformation.

10 39. The method of claim 4 or 16, wherein said DNA segments comprises plasmids.

40. The method of claim 39, wherein said DNA segments are further defined as comprising an expression cassette isolated from said plasmids.

15 41. The method of claim 4 or 16, wherein said DNA segments comprise an exogenous gene encoding a selected trait.

42. The method of claim 41, wherein said DNA segments are further defined as comprising a promoter and 3' region operatively linked to said exogenous gene.

20

43. The method of claim 42, wherein the recipient cells are transformed with at least a second exogenous gene.

25

44. The method of claim 43, wherein at least two exogenous genes are positioned on the same DNA segment, and recipient cells are contacted with said segment.

45. The method of claim 43, wherein said exogenous gene comprises a selectable or screenable marker gene.

30 46. A method of transforming a plant comprising:

(a) preparing a microprojectile composition comprising from 1 ng to 2000 ng of DNA per each 1.8 mg of starting microprojectiles;

- (b) contacting recipient plant cells with said microprojectile composition;
- (c) regenerating plants from recipient cells which have received said DNA;
- (d) identifying a fertile transgenic plant the genome of which has been augmented relative to that of the corresponding nontransgenic recipient plant through the stable introduction of said DNA.

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47. The method of claim 46, wherein said microprojectile composition is further defined as comprising from 2.5 ng to 1000 ng of DNA per each 1.8 mg of starting microprojectiles.

10 48. The method of claim 47, wherein said microprojectile composition is still further defined as comprising from 2.5 ng to 750 ng of DNA per each 1.8 mg of starting microprojectiles.

15 49. The method of claim 48, wherein said microprojectile composition is still further defined as comprising from 2.5 ng to 500 ng of DNA per each 1.8 mg of starting microprojectiles.

20 50. The method of claim 49, wherein said microprojectile composition is still further defined as comprising from 2.5 ng to 250 ng of DNA per each 1.8 mg of starting microprojectiles.

51. The method of claim 50, wherein microprojectile composition is still further defined as comprising from 2.5 ng to 100 ng of DNA per each 1.8 mg of starting microprojectiles.

25 52. The method of claim 51, wherein microprojectile composition is still further defined as comprising from 2.5 ng to 50 ng of DNA per each 1.8 mg of starting microprojectiles.

53. The method of claim 52, wherein said DNA comprises plasmids.

30 54. The method of claim 53, wherein said DNA is further defined as comprising an expression cassette isolated from said plasmids.

55. The method of claim 46, wherein said DNA comprises an exogenous gene encoding a selected trait.

56. The method of claim 55, wherein said DNA is further defined as comprising a promoter and 3' region operatively linked to said exogenous gene.

57. The method of claim 56, wherein the recipient cells are transformed with at least a second exogenous gene.

10 58. The method of claim 57, wherein at least two exogenous genes are positioned on the same DNA segment, and recipient cells are contacted with said segment.

59. The method of claim 58, wherein the exogenous gene comprises a selectable or screenable marker gene.

15 60. The method of claim 46, wherein the plant is a monocot.

61. The method of claim 60, wherein said monocot is selected from the group consisting of maize, rice, wheat, barley, oats, rye, millet, sorghum, sugarcane and turfgrass.

20 62. The method of claim 61, wherein the monocot is maize.

63. The method of claim 46, wherein the plant is a dicot.

25 64. The method of claim 63, wherein said dicot is selected from the group consisting of cotton, soybean, tomato, potato, citrus, and tobacco.

65. The method of claim 64, wherein the dicot is soybean.

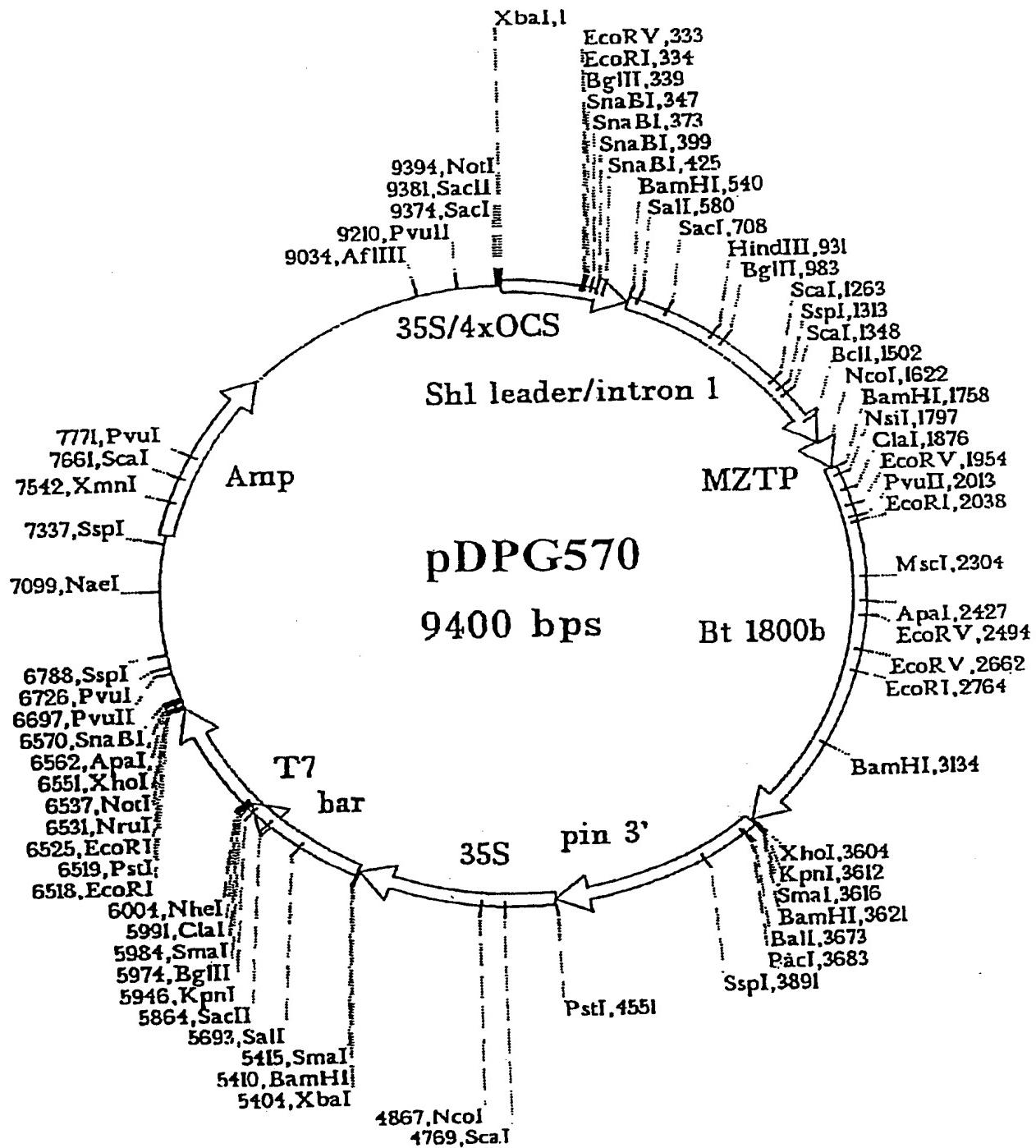


FIG. 1

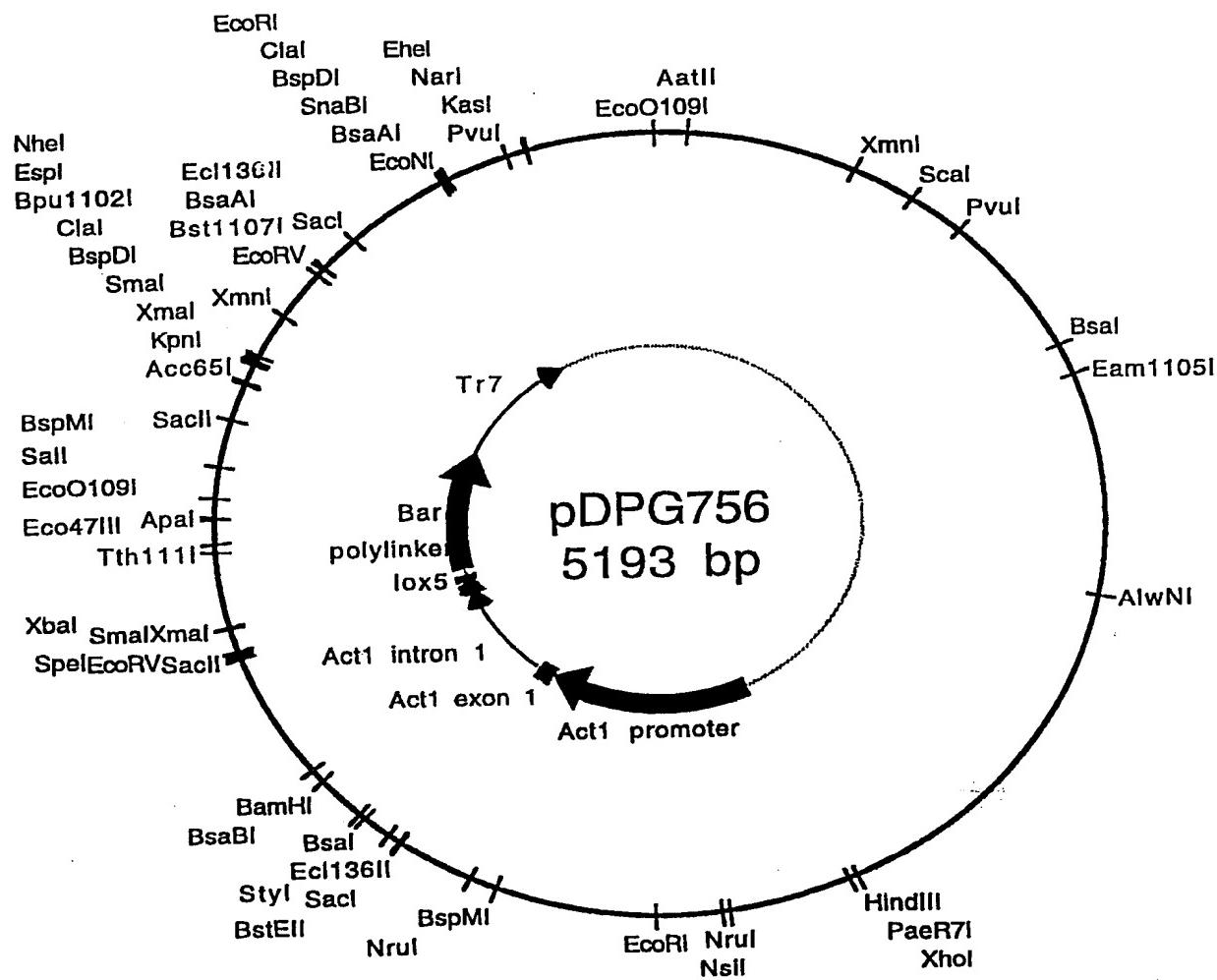


FIG. 2